REMARKS

As a preliminary matter, attention is directed to the Petition For Extension of Time of two months included herewith, which includes authorization to charge fees to the deposit account of Pfizer Inc.

Attention is also directed to the Request for Continuing Examination (RCE) also submitted herewith.

No amendments to the claims have been made.

In the discussion that follows, reference is made on occasion to text appearing in the published US application 2003/0186952 A1, both by column and line number and by published paragraph, i.e., the bracketed paragraph number taken from the publication.

Claims 1, 6 and 13 continue to be rejected under 35 USC 112, first paragraph, the Examiner having contended that the specification does not reasonably provide enablement for all cholesteryl ester protein inhibitors and all concentration-enhancing polymers. The examiner contended that HPMCAS, HPMCP, PVP, CAT, CAP, HPMC are not shown in the specification as being concentration-enhancing, and suggested that the claims recite the disclosed concentration-enhancing polymers in a Markush language.

Applicants' continue to traverse the rejection on the same basis as in their previous response, that the rejection is misplaced both factually and legally. Applicants previously directed the Examiner to their Examples. Applicants additionally direct the Examiner to paragraphs [1061] to [1091] of their specification, which paragraphs constitute an entire section dealing with (and entitled) "Concentration-Enhancing Polymers". Numerous classes and species of concentration-enhancing polymers in addition to those noted by Applicants in their examples are disclosed including, for example, all of those mentioned by the Examiner in Paragraph 3 of the Office Action, and all are disclosed specifically as being concentration-enhancing. See paragraph [1067] which discloses PVP, paragraph [1074] for HPMC, and paragraph [1077] for HPMCAS, HPMCP, CAP, and CAT. Many, many more classes of concentration-enhancing polymers and individual species are disclosed and described in that section. Even if the polymers recited are not exhaustive in the sense that every possible concentration-enhancing polymer is included, it is not required for an applicant to provide an exhaustive list. See, for example, In re Kamal, 158 USPQ 320, 323 (CCPA 1968), quoting In re Grimme, 124 USPQ 499, 501 (CCPA 1960).

> It is manifestly impracticable for an applicant who discloses a generic invention to give an example of every such species. It is sufficient if the disclosure teaches those skilled in the art what the invention is and how to practice it.

So far as the CETP inhibitors per se are concerned, Applicants have abundantly exemplified classes and a plethora of individual species in the specification, from paragraphs [0044] to [1060]. This is truly a comprehensive disclosure. Moreover, the invention is not dependent on the structure of the particular CETP inhibitor. Applicants in fact disclosed, as part of paragraph [0046] that the invention is applicable to CETP inhibitors as a class:

> ...Thus, the hydrophobic and insoluble nature of CETP inhibitors **as a class** pose a particular challenge for oral delivery. Achieving therapeutic drug levels in the blood by oral dosing of practical quantities of drug generally requires a large enhancement in drug concentrations in the gastrointestinal fluid and a resulting large enhancement in bioavailability. Such enhancements in drug concentration in gastrointestsinal fluid typically need to be at least about 10-fold and often at least about 50-fold or even at least about 200-fold to achieve desired blood levels. Surprisingly, the dispersions of the present invention have proven to have the required large enhancements in drug concentration and bioavailability. [Paragraph 0046, last 12 lines, Emphasis supplied]

Thus it is respectfully requested that the rejection be withdrawn. In addition to their examples, Applicants devoted a wealth of disclosure to describing the many embodiments, including many CETP inhibitors and concentration-enhancing polymers, that are possible within the scope of the invention. The Examiner has provided no basis as to why the invention cannot be practiced as broadly as claimed. It is respectfully submitted that there simply is no basis for deeming the invention non-enabled.

Claims 1-5, 13-20 and 22-25 continued to be rejected under 35 USC 102(b) as being anticipated by Miyajima et al. (US 4,983,593). The Examiner's restated her contention that NZ-105, the lone compound disclosed in Miyajima, is a CETP inhibitor. The Examiner stated, in pertinent part:

> 6. Applicants' arguments filed 01/14/05 have been fully considered but they are not persuasive.
>
> Kitahara discloses the reduction of VLDL by efonidipine and Toyoda discusses the benefit of NZ-105 (efonidipine) on atherosclerosis as admitted by applicants. It is known in the art that VLDL and low HDL are associated with increased risk for atherosclerosis and coronary artery disease (see teaching reference, US 6,369,075, column 4, lines 25-34). Thus the NZ-105 of Miyajima is a CETP. The instant claims do not exclude NZ-105 (efonidipine).

The Examiner is asked to reconsider her position in respect of both Kitahara and Toyoda, the abstracts she relied upon to support the contention that NZ-105 is a CETP inhibitor.

In respect of Kitahara, enclosed herewith as Exhibit A is a copy of Steinberg et al., Vol. 262, No. 7, pp3118-3122 (1987). Also enclosed as Exhibit B is a copy of Agellon et al., Vol. 266, No. 17, pp. 10796-10801 (1991). Exhibit A supports that J774 macrophages, the macrophages disclosed as being used by Kitahara, are mouse macrophages as demonstrated, for example, by the title. Exhibit B explicitly states the following in the right hand column of page 10796, lines 2-5.

> In this report, we describe the development of transgenic mice that express human plasma CETP. The mouse is especially suited for these studies since this species normally lacks plasma CE transfer activity (6).

See also the abstract of Barter et al, Exhibit C hereto, discussed below, which also highlights that mice do not contain CETP activity. The above statement confirms that mice do not have CETP, meaning that they do not produce CETP unless deliberately modified to do so. It is not possible for NZ-105 to inhibit CETP in a species that does not contain it, and for that reason Kitahara does not support NZ-105 being a CETP inhibitor. Kitahara in fact states that "...efonidipine suppresses cholesterol ester deposition ...mainly through elevation of the cellular cyclic AMP level". CETP inhibition is unrelated to elevating cyclic AMP levels. Kitahara, therefore, provides no basis for the Examiner's contention that NZ-105 is a CETP inhibitor. Kitahara et al. disclose nothing otherwise relating to CETP inhibition and, like Toyoda et al., as discussed below, simply note that it is a calcium antagonist. Calcium antagonists are known to work mechanistically by lowering blood pressure, an effect unrelated to CETP inhibition.

As to Toyoda, Applicants previously highlighted that CETP inhibition results in altering plasma lipid levels. See Applicants' introductory remarks in the specification:

> This invention relates to cholesteryl ester transfer protein (CETP) inhibitors...and the use of such inhibitors to elevate certain plasma lipid levels, including high density lipoprotein (HDL)-cholesterol and to lower certain other plasma lipid levels, such as low density lipoprotein (LDL)-cholesterol and triglycerides and accordingly to treat diseases which are affected by low levels of HDL cholesterol and/or high levels of LDL-cholesterol and triglycerides, such as atherosclerosis

> and cardiovascular diseases in certain mammals (i.e., those which have CETP in their plasma) including humans. (Specification, page 1, lines 10-21)

It is Applicants' position that Toyoda discloses nothing that would support NZ-105 being a CETP inhibitor, and in fact supports that it is not. The art recognizes that CETP inhibitors act by affecting plasma lipid levels. See, for example, Barter et al., attached hereto as Exhibit C, a review article entitled "Cholesteryl Ester Transfer Protein; A Novel Target for raising HDL and inhibiting Atherosclerosis". The article clearly highlights that CETP (and its inhibition) acts by affecting lipid levels. Toyoda, in distinct contrast, discloses that efonidipine (NZ-105) did not affect plasma lipids:

> These results suggest that NZ-105 may suppress the development of atherosclerosis without affecting the plasma lipids. (Toyoda et al., last sentence)

The Examiner stated that "Toyoda discusses the benefit of NZ-105 (efonidipine) on atherosclerosis..." and that "It is known in the art that VLDL and low HDL are associated with increased risk for atherosclerosis and coronary artery disease..." and concluded that "NZ-105 of Miyajima is a CETP...". That reasoning is not understood since it is possible for other classes of compounds such as statins and fibrates to have beneficial effects on atherosclerosis through mechanisms unrelated to CETP inhibition. In summary, the Toyoda abstract itself supports that NZ-105 is not a CETP inhibitor, for the following reasons.

(1) The Toyoda abstract itself states that NZ-105 has no effect on plasma lipids. See the last three lines of the abstract which state "...These results suggest that NZ-105 may suppress the development of atherosclerosis without affecting the plasma lipids". The fact that NZ-105 does not affect plasma lipids means that NZ-105 is not inhibiting CETP. The mechanism of CETP inhibition, by definition, requires an effect on plasma lipids.

(2) The only utility attributed to NZ-105 by Toyoda is that it is a dihydropyridine calcium antagonist, a utility associated with lowering blood pressure. Although high blood pressure (hypertension) is a well-known independent risk factor for developing atherosclerosis, that factor is independent of any action on CETP. Thus the only utility attributed by Toyoda to NZ-105, namely that of being a calcium antagonist, is a utility unrelated to affecting plasma lipids, which Toyoda et al. disavow in any event.

(3) Toyoda says nothing about CETP inhibition or CETP inhibitors. At most, Toyoda uses the phrase "cholesterol ester" in discussing cholesterol ester reduction in the aorta, but offers no basis that the reduction is due to cholesterol ester transfer protein (CETP) inhibition.

Miyajima does not otherwise disclose or suggest that CETP inhibitors generally would have utility in the instant invention. Thus Miyajima does not disclose all elements of the claimed invention, hence cannot anticipate. Withdrawal of the rejection is accordingly respectfully requested.

Claims 6-12 continue to be rejected under 35 USC 103(a) as being unpatentable over Miyajima et al (US 4,983,593) in view of Nakamichi et al (US 5,456,923). The Examiner based the rejection in part on her contention that NZ-105 is a CETP inhibitor. Applicants respectfully submit that NZ-105 is not, based on the discussion above, and request that the Examiner reconsider the rejection based on the evidence submitted herewith (i.e., Applicants' discussion and the accompanying Exhibits). Applicants continue to traverse the rejection on the basis that it is (1) based on hindsight and (2) not supported by the references.

One of the underpinnings of this invention is that CETP inhibitors constitute a class of compounds that has poor aqueous solubility, and that has correspondingly low bioavailability. Much of the technology directed to improving the solubility and/or concentration of drugs generally has had only limited success when applied to CETP inhibitors. In this regard, and to provide useful background information against which the non-obviousness of Applicants' invention can be assessed, the following text is a quotation taken directly from the specification because it offers insights into the problems that Applicants have solved:

> However, it has proven difficult to formulate CETP inhibitors for oral administration such that therapeutic blood levels are achieved. CETP inhibitors in general possess a number of characteristics which render them poorly bioavailable when dosed orally in a conventional manner. CETP inhibitors tend to be quite hydrophobic and extremely water insoluble, with solubility in aqueous solution of usually less than about 10 μg/ml and typically less than 1 μg/ml. Often, the aqueous solubility of CETP inhibitors is less than 0.1 μg/ml. Indeed the solubility of some CETP inhibitors is so low that it is in fact difficult to measure. Accordingly, when CETP inhibitors are dosed orally, concentrations of CETP inhibitor in the aqueous environment of the gastrointestinal tract tend to be extremely low, resulting in poor absorption from the GI tract to blood. The hydrophobicity of CETP inhibitors not only leads to low equilibrium aqueous solubility but also tends to make the drugs poorly wetting and slow to dissolve, further reducing their tendency to dissolve and be absorbed from the gastrointestinal tract. This combination of characteristics has resulted in the

bioavailability for orally dosed conventional crystalline or amorphous forms of CETP inhibitors generally to be quite low, often having absolute bioavailabilities of less than 1%.

Various attempts have been made to improve the aqueous concentration of CETP inhibitors , but generally have met with limited success. At the outset, most methods aimed at enhancing aqueous concentration and bioavailability of low-solubility drugs offer only moderate improvements. Such improvements generally lead to enhancements in aqueous concentration on the order of from one to seven fold. In addition, the enhancement may be short-lived, with the drug concentration returning to the equilibrium concentration within ten to 40 minutes. Such small, short-lived concentration enhancements have led to even lower levels of bioavailability enhancement when tested *in vivo* via oral administration. Thus, when conventional dosage forms of low-solubility drugs are tested *in vivo* via oral administration, bioavailability enhancements are typically on the order of 2-fold to 4-fold or less. For CETP inhibitors having low absolute bioavailabilities, such small improvements are insufficient to allow convenient oral dosing of CETP inhibitors; that is, dosage forms having a convenient size and frequency of dosing.

Moreover, some standard methods for improving the concentration of pharmaceuticals in aqueous solution have proven inadequate when applied to CETP inhibitors. For example, even pre-dissolving the CETP inhibitor in a water miscible solvent such as polyethylene glycol followed by delivery as a solution to an aqueous environment of use has failed to raise the aqueous concentration of CETP inhibitor to an acceptable level. [Page 3, line 8 to page 4, line 21 of Applicants' specification]

Neither Miyajima nor Nakamichi discloses CETP inhibitors, as discussed above. Consequently, neither reference is capable of disclosing that any CETP inhibitor, much less CETP inhibitors as a class, would have a greatly improved maximum concentration by virtue of being formed as a solid amorphous dispersion by any of Applicants' claimed methods. Neither reference discloses or suggests that Applicants compositions would produce such good results, namely that a 10-fold maximum concentration (i.e., relative to a control containing no polymer) could be achieved with CETP inhibitors in a solid amorphous dispersion. Combining the references does nothing to change the conclusion of non-obviousness. Nakamichi relates to using twin screw extrusion, but is silent about CETP inhibitors for use with that method. Miyajima mentions a number of methods for combining a single compound, NZ-105, with HPMCAS, but says nothing relating to any CETP inhibitor individually or to CETP inhibitors as a class, much less anything that would render a 10-fold increase in CETP inhibitor maximum concentration obvious. The Examiner has provided no basis whereby one of ordinary skill in the art would find it obvious to use Applicants' methods to produce compositions that increase the maximum concentration of CETP inhibitors by such a large factor.

Indeed, because the references fail to disclose CETP inhibitors, their problematic low aqueous solubility, or any way of achieving a 10-fold improvement in aqueous CETP inhibitor maximum concentration, the only way that Applicants' invention could possibly be obvious is through an impermissible hindsight analysis. The Examiner appears to have imputed the missing teachings, individually and in combination, to the references when, in fact, they and their combination were disclosed only by Applicants. It is respectfully submitted that, by that analysis, the Examiner has fallen "victim to the insidious effect of a hindsight syndrome wherein that which the invention taught is used against its teacher." *W.L. Gore & Associates v. Garlock, Inc.*, 721 F.2d. 1540, 1553 (Fed. Cir. 1983). It is otherwise well-accepted law that it is impermissible to use the inventor's disclosure as a road map for selecting and combining prior art disclosures. Grain Processing Corp. v. American Maize-Products Corp., 5 USPQ2d, 1788 (Fed. Cir. 1988). That law is even more applicable when Applicants' own disclosure has been used to supply disclosure. Again, neither Miyajima nor Nakamichi discloses any CETP inhibitor or suggests any method for improving the maximum concentration of CETP inhibitors in a use environment by 10-fold. Clearly, given the factual setting in which the invention as a whole was made, and based on the legal standards reviewed above, Applicants' invention is non-obvious, hence patentable.

Accordingly, it respectfully requested that the §103 rejection over Miyajima in view of Nakamichi be withdrawn.

No additional issues are seen to be outstanding, and in view of the foregoing comments and amendments, this case is believed to be in condition for allowance. A Notice of Allowance is courteously solicited.

Respectfully submitted,

Date: SEPT. 29, 2005

James T. Jones
Attorney for Applicant
Reg. No. 30,561

Pfizer Inc
Patent Department
Eastern Point Road
Groton, CT 06340
(860) 441-4903

Exhibit A

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 262, No. 7, Issue of March 5, pp. 3118–3122, 1987
Printed in U.S.A.

Extracellular ATP^{4-} Promotes Cation Fluxes in the J774 Mouse Macrophage Cell Line*

(Received for publication, July 23, 1986)

Thomas H. Steinberg‡ and Samuel C. Silverstein

From the Rover Physiology Laboratory of the Department of Physiology and Cellular Biophysics, Columbia University College of Physicians and Surgeons, New York, New York 10032

Extracellular ATP stimulates transmembrane ion fluxes in the mouse macrophage cell line J774. In the presence of Mg^{2+}, nonhydrolyzable ATP analogs and other purine and pyrimidine nucleotides do not elicit this response, suggesting the presence of a specific receptor for ATP on the macrophage plasma membrane. One candidate for such a receptor is the ecto-ATPase expressed on these cells. We, therefore, investigated the role of this enzyme in ATP-induced $^{86}Rb^+$ efflux in J774 cells. The ecto-ATPase had a broad nucleotide specificity and did not hydrolyze extracellular ATP in the absence of divalent cations. $^{86}Rb^+$ efflux was not blocked by inhibition of the ecto-ATPase and did not require Ca^{2+} or Mg^{2+}. In fact, ATP-stimulated $^{86}Rb^+$ efflux was inhibited by Mg^{2+} and correlated with the availability of ATP^{4-} in the medium. In the absence of divalent cations, the slowly hydrolyzable ATP analogs adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP) and adenosine 5′-O-(3-thio)triphosphate (ATP-γ-S) also stimulated $^{86}Rb^+$ efflux, albeit at higher concentrations than that required for ATP^{4-}. Exposure of J774 cells to 10 mM ATP for 45 min caused death of 95% of cells. By this means we selected variant J774 cells that did not exhibit $^{86}Rb^+$ efflux in the presence of extracellular ATP but retained ecto-ATPase activity.

These results show that the ecto-ATPase of J774 cells does not mediate the effects of ATP on these cells; that ATP^{4-} and not $MgATP^{2-}$ promotes $^{86}Rb^+$ efflux from these cells; and that hydrolysis of ATP is not required to effect this change in membrane permeability. These findings suggest that J774 cells possess a plasma membrane receptor which binds ATP^{4-}, AMP-PNP, and ATP-γ-S, and that the ecto-ATPase limits the effects of ATP on these cells by hydrolyzing $MgATP^{2-}$.

While the pivotal role of adenosine triphosphate (ATP) in intermediary metabolism has been appreciated since the 1930s, recognition of ATP as a transmitter of intercellular signals has emerged only recently. Both ATP and adenosine have been identified as transmitter substances and regulatory molecules in the nervous system (1). Receptors for ATP and adenosine have been distinguished from one another by relative substrate affinities (2), and ATP-induced changes in membrane conductance have been detected in individual neurons (3).

Adrenal chromaffin cells (4), platelets (5), and endothelial cells (6) release ATP, and a variety of cells, including hepatocytes (7), mast cells (8), phagocytes (9), and several transformed cell lines (10–12) respond to micro- or millimolar concentrations of exogenous ATP with changes in membrane ion fluxes, cytosolic Ca^{2+} levels, and functional concomitants that depend on cell type. The nature of the ATP receptors of these cells and the ways in which external ATP affects membrane and cytosolic events are unknown. Ectoenzymes that bind ATP, including ATPases (13, 14) and protein kinases (15, 16), are present on a number of cells and are candidates for the ATP receptor. Becker and Henson (9) in their studies of ATP-mediated lysosomal enzyme secretion of rabbit polymorphonuclear leukocytes and McCord *et al.* (17) in their studies on ATP-induced inhibition of phagocytosis in human polymorphonuclear leukocytes suggested that these effects of ATP are produced by an ecto-ATPase.

However, the effects of ATP on rat mast cells appear not to involve ecto-ATPase activity. Dahlquist and Diamant (18) and Cockcroft and Gomperts (8) showed that histamine release from these cells is mediated by ATP^{4-} rather than $MgATP^{2-}$. Since $MgATP^{2-}$ is the substrate for all known ATPases, the putative "ATP^{4-} receptor" of these cells is unlikely to be an ATPase.

In the mouse macrophage cell line J774, extracellular ATP induces plasma membrane depolarization, Na^+ influx, K^+ efflux, an increase in cytosolic free Ca^{2+}, and inhibition of Fc receptor-mediated phagocytosis (19). In the presence of Mg^{2+}, millimolar concentrations of ATP are required to induce these responses, and ATP analogs and nucleotides other than ATP are ineffective. Since ATP cannot enter these cells, the specificity of these ATP-induced events suggests that ATP interacts with a specific receptor on the J774 cell membrane. J774 macrophages possess a plasma membrane ecto-ATPase that hydrolyzes external ATP and is, therefore, a possible mediator of these ATP-stimulated events.

In the present study we have investigated the relationship between ATP-stimulated ion fluxes and the ecto-ATPase present on J774 cells. Hydrolysis of exogenous ATP was inhibited by GTP and ITP while ATP-mediated $^{86}Rb^+$ efflux was not. Ecto-ATPase activity required divalent cations in the medium; in contrast, ATP-mediated $^{86}Rb^+$ efflux was inhibited by Mg^{2+} and correlated with the concentration of ATP^{4-} present in the medium. In addition, we observed that prolonged exposure of J774 cells to 10 mM ATP led to cell death. J774 cells selected for resistance to ATP did not efflux $^{86}Rb^+$ in the presence of extracellular ATP but displayed ecto-ATPase activity. We conclude that the ATP receptor of J774

* This work was supported by a Clinical Scientist Research Fellowship from the Damon Runyon-Walter Winchell Cancer Fund (to T. H. S.), a generous gift from Samuel Rover, and by United States Public Health Service Grant AI 20516. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Physiology, Columbia University College of Physicians and Surgeons, 630 W. 168 St., New York, NY 10032.

binds uncomplexed ATP^{4-} rather than $MgATP^{2-}$, and the ecto-ATPase serves to limit the effects of extracellular ATP.

MATERIALS AND METHODS

Chemicals—ATP (special grade), GTP, ITP, UTP, CTP, and ATP-γ-S[1] were purchased from Boehringer Mannheim. AMP-PCP and AMP-PNP were purchased from Pharmacia P-L Biochemicals. 2′-Deoxyadenosine 5′-triphosphate (dATP) was bought from Sigma; [γ-^{32}P]ATP, [γ-^{32}P]GTP, and ^{86}RbCl were bought from New England Nuclear.

Cells and Media—The J774 mouse macrophage cell line was grown in spinner cultures in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (HIFBS). Cells were plated for 6 h or overnight in 16-mm tissue culture wells at a density of 5–10 × 10^5 cells/well for experiments requiring adherent cells. Cell viability was assessed by trypan blue exclusion (20).

Ecto-ATPase Activity—Cells were plated in 16-mm wells in Dulbecco's modified Eagle's medium with 10% HIFBS. The medium was removed and "ATPase buffer" (135 mM NaCl, 5 mM KCl, 3.5 mM $MgCl_2$, 10 mM HEPES, pH 7.4) or MEM with 10 mM HEPES (pH 7.4), divalent cations, nucleotides, and ATP at the concentrations indicated was added along with 2 μCi/ml [γ-^{32}P]ATP. 50-μl samples were removed at appropriate intervals and added to microcentrifuge tubes containing 1 ml of 20% (w/v) acid-washed activated charcoal in 10% trichloroacetic acid. The microcentrifuge tubes were spun at 10,000 rpm for 5 min to sediment the charcoal with adsorbed ATP, and 100 μl of supernatant containing released $^{32}P_i$ was added to scintillation vials. Cerenkov radiation was measured in a scintillation counter (LKB Rack-Beta). The total radiolabel in 50 μl of medium also was measured. Cells were dissolved with 0.05% Triton X-100, and the quantity of cell protein in each tissue culture well was assayed by the method of Lowry *et al.* (21). ATPase activity was expressed as nmol of ATP hydrolyzed/mg of cell protein/min.

Ecto-GTPase Activity—Hydrolysis of GTP was measured using the ATPase assay with [γ-^{32}P]GTP substituted for [γ-^{32}P]ATP.

Kinetic Analysis—We assessed the consistency of ATPase data by constructing "direct linear" plots as described by Cornish-Bowden (22). The kinetic parameters K_m and V_{max} were derived from linear regression analyses of s/v *versus* s (Hanes plot). The linear equation so defined has a slope equal to $1/V_{max}$ and an x intercept equal to $-K_m$.

Rubidium Efflux—J774 cells (10^6 cells/16-mm well) were incubated in 250 μl of MEM with 2% HIFBS and 5 μCi/ml ^{86}RbCl for 90 min. Each well was washed twice with Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PD), and 500 μl of medium (MEM or buffered saline) with the appropriate nucleotides and divalent cations was added. 50-μl aliquots of medium were removed at 0, 5, and 10 min and added to scintillation vials. Radioactivity was determined in a β counter (LKB Rack-Beta). Cells were solubilized in 0.05% Triton X-100 in H_2O, and residual intracellular $^{86}Rb^+$ was measured. To determine total initial intracellular $^{86}Rb^+$, cells were incubated with ^{86}RbCl, washed, solubilized in 0.05% Triton X-100, and radioactivity was measured. Cell viability was assessed in parallel wells by trypan blue exclusion.

RESULTS

The Ecto-ATPase of J774 Macrophages Is a Nucleotidase—Adherent J774 cells hydrolyzed exogenous ATP at 37 °C with an apparent K_m of 365 ± 106 μM and V_{max} of 32 ± 4 nmol/mg of protein/min (Fig. 1). Ecto-ATPase activity was constant over the pH range of 5.5–8.5 (data not shown). Alkaline phosphodiesterase, present on the plasma membrane of J774 cells (23), did not contribute significantly to hydrolysis of exogenous ATP, since addition of 5 mM paranitrophenyl phosphate, a substrate for this phosphodiesterase, did not significantly reduce ATP hydrolysis (Fig. 1).

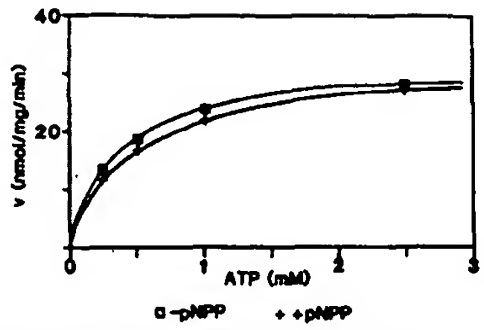

FIG. 1. **Ecto-ATPase activity of J774 cells.** 5 × 10^5 cells were plated per 16-mm tissue culture well. MEM with 2% HIFBS, 10 mM HEPES (pH 7.4), 2 μCi/ml [γ-^{32}P]ATP, and varying concentrations of MgATP were added, and samples were processed as described under "Materials and Methods." □, without 5 mM paranitrophenyl phosphate (*PNPP*); +, with 5 mM paranitrophenyl phosphate.

TABLE I

Nucleotides and ATP analogs inhibit hydrolysis of exogenous ATP

J774 cells (5 × 10^5 cells/ml) were suspended in "ATPase buffer" containing 135 mM NaCl, 5 mM KCl, 3.5 mM $MgCl_2$, and 10 mM HEPES (pH 7.4) with 1 mM MgATP, 2 μCi/ml [γ-^{32}P]ATP, and other competing nucleotides as listed. ATPase activity was measured at 30 min as described under "Materials and Methods." Results are expressed as percent of hydrolysis in the absence of competing nucleotide.

Inhibitor	Control ATPase activity
	%
10 mM GTP	39 ± 10
10 mM ITP	31 ± 10
10 mM CTP	40 ± 13
10 mM AMP-PCP	37 ± 2

TABLE II

Comparison of ecto-ATPase and ecto-GTPase activities of J774

J774 cells (5 × 10^5/ml) were suspended in medium with 10 mM HEPES, 1 mM MgATP, or 1 mM MgGTP and 2 μCi/ml [γ-^{32}P]ATP or [γ-^{32}P]GTP. ATP or GTP hydrolysis was measured as described and expressed as percent of total radiolabel hydrolyzed at each time point.

	15 min	30 min	60 min
	%	%	%
ATP hydrolysis	13.2	26.6	44.9
GTP hydrolysis	16.3	34.1	57.8

The effects of extracellular ATP on J774 macrophages were reported to be mediated only by ATP and not by other nucleotides or ATP analogs (19). We, therefore, tested the ability of nucleotides other than ATP to block ATP hydrolysis. 10 mM GTP, ITP, or CTP inhibited hydrolysis of 1 mM ATP by 60–69% (Table I). The nonhydrolyzable ATP analog AMP-PCP similarly inhibited ATP hydrolysis. These findings show that both purine and pyrimidine nucleoside triphosphates can compete with ATP for the ecto-ATPase; they suggest that the ecto-ATPase also can hydrolyze nucleotides other than ATP. We confirmed this hypothesis by showing that hydrolysis of exogenous [γ-^{32}P]GTP and [γ-^{32}P]ATP occurred at similar rates (Table II). Furthermore, unlabeled ATP inhibited the hydrolysis of GTP in a concentration-dependent fashion (data not shown). Thus the ecto-ATPase of J774 macrophages might best be termed an ectonucleotidase.

ATP-induced Rubidium Efflux from J774 Cells Is Not Inhibited by Other Nucleotides—ATP causes J774 macrophages to release intracellular $^{86}Rb^+$ (19). The $K_{1/2}$ for MgATP was approximately 1.5 mM; 1 mM MgATP stimulated little efflux,

[1] The abbreviations used are: ATP-γ-S, adenosine 5′-*O*-(3-thio)triphosphate; AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate; AMP-PCP, adenosine 5′-(β,γ-methylene)triphosphate; HIFBS, heat-inactivated fetal bovine serum; MEM, Eagle's minimal essential medium; PD, Dulbecco's phosphate-buffered saline without divalent cations; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

while 2 mM MgATP induced almost total release of the intracellular $^{86}Rb^+$ (Fig. 2). If ATP hydrolysis via the ecto-ATPase mediates ATP-induced $^{86}Rb^+$ efflux, then nucleotides that compete with ATP for the ecto-ATPase should inhibit this efflux. Therefore, we measured the efflux of $^{86}Rb^+$ from J774 cells in the presence of 10 mM GTP or ITP and either 1 or 2 mM ATP. In these experiments, all nucleotides were added with equimolar Mg^{2+} so that competition for divalent cations would not influence the results. The addition of 10 mM GTP had no effect on $^{86}Rb^+$ efflux elicited by 2 mM ATP (Fig. 2). 10 mM GTP alone, or in combination with 1 mM ATP, did not stimulate $^{86}Rb^+$ efflux. 10 mM ITP did not promote $^{86}Rb^+$ efflux, but when 10 mM ITP was added to 1 mM ATP, $^{86}Rb^+$ efflux was markedly enhanced. It is not clear why GTP and ITP affected ATP-mediated $^{86}Rb^+$ efflux differently, but neither of these results is consistent with the marked reduction in ATP-mediated $^{86}Rb^+$ efflux one would expect if ATP hydrolysis was required for $^{86}Rb^+$ efflux to occur.

Ecto-ATPase Activity of J774 Requires Magnesium or Calcium—Mg^{2+} binds ATP^{4-} with high affinity, forming a bidentate coordination complex of 1:1 stoichiometry involving the β- and γ-phosphates of ATP (24). The substrate for most ATPases and other enzymes that utilize ATP is this $MgATP^{2-}$ complex rather than free ATP^{4-}. When ecto-ATPase activity of J774 cells was measured in the presence of 1 mM EDTA in buffered saline solution, hydrolysis of ATP was reduced to 5% of hydrolysis in the presence of Ca^{2+} and Mg^{2+} (Table III). The ecto-ATPase of J774 did not show an absolute requirement for Mg^{2+}, since Ca^{2+} was equally active in supporting ATPase activity.

ATP-mediated Rubidium Efflux Is Inhibited by Magnesium—In contrast to ecto-ATPase activity, ATP-stimulated $^{86}Rb^+$ efflux occurred in the absence of divalent cations. Neither 1 mM EDTA nor 5 mM Mg^{2+} promoted $^{86}Rb^+$ efflux in the absence of ATP, and efflux was nearly complete under all conditions in the presence of 5 mM ATP (Fig. 3). While 2 mM ATP induced maximal efflux in cells incubated in buffered saline with or without EDTA, addition of 5 mM Mg^{2+} reduced $^{86}Rb^+$ efflux in the presence of 2 mM ATP. These results show that ATP promotes $^{86}Rb^+$ efflux under conditions which inhibit ecto-ATPase activity; they suggest that the putative membrane receptor for ATP^{4-} does not require Mg^{2+} as does the ecto-ATPase.

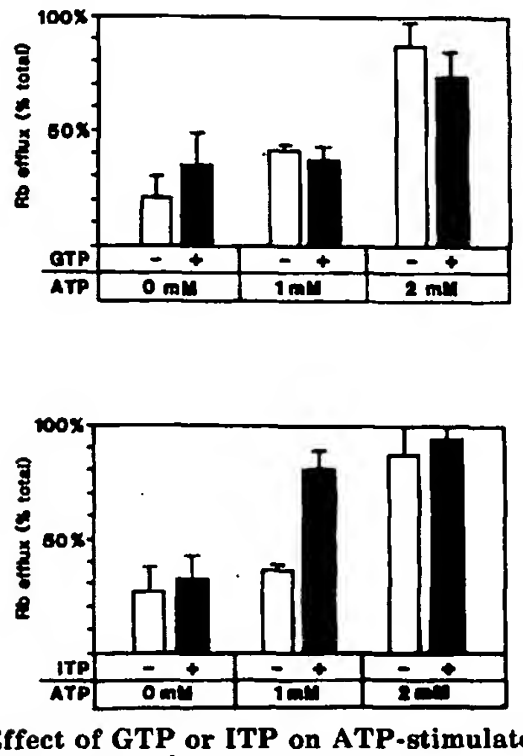

FIG. 2. **Effect of GTP or ITP on ATP-stimulated $^{86}Rb^+$ efflux from J774 cells.** 10^6 cells were plated per 16-mm tissue culture well, incubated in MEM with 2% HIFBS and 5 μCi/ml $^{86}RbCl$ for 90 min, washed twice, and medium with 0, 1, or 2 mM ATP, 10 mM MgGTP, or MgITP was added as indicated. Samples were processed as described under "Materials and Methods." Results are expressed as percent of total intracellular $^{86}Rb^+$ that has left the cell.

TABLE III

Ecto-ATPase activity of J774 requires divalent cations

10^6 J774 cells were plated in each 16-mm tissue culture well. ATP hydrolysis was measured after 20 min of incubation in ATPase buffer with divalent cations or EDTA as described.

Condition	ATP hydrolysis
	nmol/mg protein/min
5 mM Ca^{2+} + 5 mM Mg^{2+}	29.2 ± 6.4
1 mM EDTA	1.4 ± 1.7
1 mM EDTA + 5 mM Ca^{2+}	21.6 ± 4.6
1 mM EGTA + 5 mM Mg^{2+}	20.4 ± 1.1

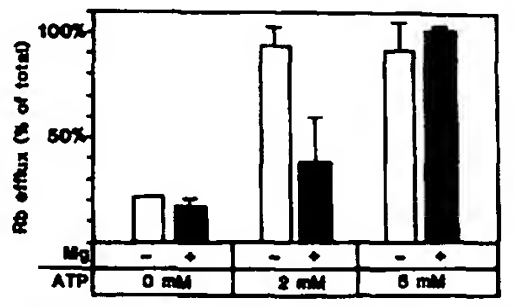

FIG. 3. **Effect of EDTA of Mg^{2+} on ATP-stimulated $^{86}Rb^+$ efflux.** J774 cells (10^6/16-mm tissue culture well) were incubated in MEM with 2% HIFBS and 5 μCi/ml $^{86}RbCl$ for 90 min. The cells were washed twice with PD and then incubated with PD containing 10 mM HEPES (pH 7.4) and 1 mM EDTA or 5 mM $MgSO_4$ in the presence of 0, 2, or 5 mM ATP. $^{86}Rb^+$ efflux was measured as described.

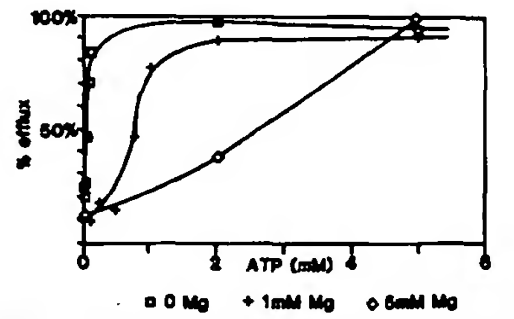

FIG. 4. **Effect of varying magnesium concentrations on ATP-induced $^{86}Rb^+$ efflux.** 10^6 cells/16-mm tissue culture well were incubated in MEM with 2% HIFBS and 5 μCi/ml $^{86}RbCl$ for 90 min. Cells were washed twice with PD and incubated in PD containing 1 mM EDTA (for 0 mM magnesium), 1 mM $MgSO_4$, or 5 mM $MgSO_4$ and ATP as indicated. $^{86}Rb^+$ efflux was measured as described.

The effect of ATP on $^{86}Rb^+$ efflux was determined in the presence of various concentrations of Mg^{2+}. As the concentration of Mg^{2+} was increased, the concentration of ATP required to stimulate $^{86}Rb^+$ efflux also increased. In the absence of all divalent cations (1 mM EDTA), 100 μM ATP caused maximal $^{86}Rb^+$ efflux in 10 min; the $K_{1/2}$ was approximately 50 μM ATP (Fig. 4). In the presence of 1 mM Mg^{2+}, maximal efflux required 1 mM ATP, and the $K_{1/2}$ was 700 μM ATP. 5 mM Mg^{2+} increased the amount of ATP necessary to cause total $^{86}Rb^+$ efflux to 5 mM, with a $K_{1/2}$ of 3 mM ATP.

ATP exists as several ionic species in neutral salt solutions containing various monovalent and divalent cations, and rigorous calculation of their concentrations must consider the equilibria of all species, including the various protonated forms of ATP as well as Ca^{2+} and Mg^{2+} complexes. Approximations of the concentration of "free" ATP^{4-} can be made under the present experimental conditions by ignoring Ca^{2+} complexes, since no Ca^{2+} was present in the medium, and by considering only the most prevalent ionic complexes. Given

the association constants for Mg^{2+} + ATP^{4-} (log K_a = 4.00) and H^+ + ATP^{4-} (log K_a = 6.95) (25), a second degree equation can be used to calculate [ATP^{4-}] and [$MgATP^{2-}$] for known values of [Mg^{2+}], [ATP], and pH (see "Appendix"). From the above data we calculated [ATP^{4-}] and [$MgATP^{2-}$] at half-maximal $^{86}Rb^+$ efflux in the presence of 1 mM Mg^{2+} and 5 mM Mg^{2+} and found that $^{86}Rb^+$ efflux correlated well with [ATP^{4-}] and poorly with [$MgATP^{2-}$]; the calculated $K_{1/2}$ of ATP^{4-} for $^{86}Rb^+$ efflux was 120 μM in the presence of 1 mM Mg^{2+} and 130 μM in the presence of 5 mM Mg^{2+}, while the corresponding $MgATP^{2-}$ concentrations were 0.54 and 2.8 mM, respectively. These results confirm that Mg^{2+} inhibits ATP-mediated $^{86}Rb^+$ efflux; they suggest that the surface molecules which bind ATP and mediate these events do not bind $MgATP^{2-}$ but instead bind the unchelated ATP^{4-}.

In the Absence of Magnesium, ATP Analogs Mediate Rubidium Efflux—In the presence of Mg^{2+}, ATP analogs and nucleotides other than ATP do not cause $^{86}Rb^+$ efflux (19). Since the putative membrane receptor that mediates cation fluxes on J774 cells binds ATP^{4-} but not $MgATP^{2-}$, it is likely that the conformation of the terminal phosphates is important in receptor ligation. One might predict, therefore, that removal of extracellular divalent cations would enhance the ability of ATP analogs to interact with this receptor, especially those analogs which have strong affinities for divalent cations. This prediction was confirmed by experiments. In the presence of 1 mM EDTA, the slowly hydrolyzable ATP analog ATP-γ-S at 1 mM concentration caused marked $^{86}Rb^+$ efflux while the nonhydrolyzable ATP analog AMP-PNP did so at 5 mM (Table IV). The amount of contaminating ATP present in the AMP-PNP preparation was measured using the lucifer-luciferase assay (26). A maximum of 0.2% ATP or 10 μM ATP in 5 mM AMP-PNP was found; therefore, contamination of this analog with ATP could not account for the efflux seen. Under the same conditions, 5 mM GTP, ITP, dATP, or AMP-PCP still had no effect on $^{86}Rb^+$ efflux (data not shown).

These results confirm that hydrolysis of substrate is not required for $^{86}Rb^+$ efflux to occur, and they are consistent with our interpretation that removal of divalent cations allows ATP to interact with a membrane receptor. One factor that may account for the higher concentrations of some ATP analogs needed to stimulate $^{86}Rb^+$ efflux is their affinity for divalent cations relative to ATP; both AMP-PCP and AMP-PNP have affinity constants for divalent cations that are 2–2.5-fold greater than that of ATP (24). Therefore, the concentration of analog uncomplexed to Mg^{2+} (*e. g.* $AMP\text{-}PNP^{4-}$) will be lower than the concentration of ATP^{4-} under similar conditions.

TABLE IV

ATP analogs stimulate rubidium efflux in the absence of divalent cations

10^6 J774 cells were plated in each tissue culture well and incubated with 5 μCi/ml $^{86}RbCl$ for 90 min. Cells were washed twice with PD, and PD with 10 mM HEPES, 1 mM EDTA, and ATP analogs as indicated was added. ^{86}Rb efflux was determined as described and expressed as efflux (percent of total) at 10 min ± S.D.

Analog	Rb efflux
	%
Control	32.8 ± 8.1
0.1 mM ATP-γ-S	35.8 ± 6.1
0.5 mM ATP-γ-S	53.3 ± 2.4
1 mM ATP-γ-S	94.4 ± 3.9
1 mM AMP-PNP	35.1 ± 5.5
2.5 mM AMP-PNP	40.8 ± 2.6
5 mM AMP-PNP	94.7 ± 4.7

Variant J774 Cells Do Not Efflux Cations When Exposed to Exogenous ATP but Maintain ecto-ATPase Activity—Because extracellular ATP causes drastic changes in the intracellular cation composition of the J774 cell (19), we reasoned that prolonged exposure to high concentrations of ATP would lead to cell death. Exposure of cells to 10 mM ATP for 15, 30, or 45 min led to death of 50, 75, or 95% of cells, respectively. J774 cells were exposed to 10 mM ATP for 45 min, and viable cells were allowed to repopulate the cultures. After four successive cycles of exposure to ATP and regrowth of surviving cells to confluence (7–10 days), extracellular ATP no longer had any appreciable effect on cell viability. The ATP-resistant cultures did not efflux $^{86}Rb^+$ in the presence of 10 mM ATP. Limiting dilution cultures of ATP-resistant cells were grown, and eight cultures arising from single cells were established. One of these cell lines, ATPR B2, was analyzed further.

The ability of extracellular ATP to induce $^{86}Rb^+$ efflux in ATPR B2 cells was examined. Exposure of these cells to 5 or 10 mM ATP for 10 min, in the presence or absence of Mg^{2+}, did not stimulate $^{86}Rb^+$ efflux above the base-line efflux seen in the absence of ATP. ATPR B2 cells are, therefore, at least 200-fold less sensitive to ATP than normal J774 cells.

However, ATPR B2 cells were able to hydrolyze extracellular ATP. Kinetic analysis of ATPR B2 ecto-ATPase activity yielded a K_m of 339 ± 87 μM, comparable to that of normal J774 cells, but a V_{max} that was 11 ± 4 nmol/mg of protein/min or one-third of the V_{max} of normal J774 cells. These data suggest that the ATPR B2 cells possess a reduced number of normal ecto-ATPase molecules.

DISCUSSION

These studies prove that the effects of external ATP on J774 cells are not induced by $MgATP^{2-}$, the substrate for ATPases, and strongly suggest that these effects are mediated by a plasma membrane receptor for ATP^{4-}. Studies of ATP-induced histamine release from rat mast cells have led Dahlquist and Diamant (18) and Cockcroft and Gomperts (8) to posit the existence of a similar ATP^{4-} receptor. Mg^{2+} inhibited ATP-stimulated histamine secretion in mast cells as it did $^{86}Rb^+$ release in macrophages. In both cell types, these events proved to be a function of the ATP^{4-} concentration. ATP-induced cation permeability in dog red blood cells also can be prevented by divalent cations (27).

The finding that ATP^{4-}, not $MgATP^{2-}$, causes $^{86}Rb^+$ efflux in J774 cells explains why millimolar ATP is required to mediate cation fluxes in the presence of Mg^{2+}. It also suggests how the ecto-ATPase might be a regulatory enzyme for ATP-induced cation fluxes even though it has a K_m that is severalfold higher than the $K_{1/2}$ of the ATP-induced effects. Because the K_m of the ecto-ATPase is 365 μM ATP, this enzyme cannot efficiently maintain the concentration of MgATP in the low micromolar range. If $^{86}Rb^+$ efflux were caused by $MgATP^{2-}$, the ecto-ATPase could not lower the concentration of $MgATP^{2-}$ sufficiently to prevent this efflux. However, because of the high affinity of Mg^{2+} for ATP^{4-}, [ATP^{4-}] will be only a fraction of [$MgATP^{2-}$] when Mg^{2+} is present in physiologic concentrations. It is this smaller [ATP^{4-}] that determines $^{86}Rb^+$ efflux. The ecto-ATPase by regulating the more abundant [$MgATP^{2-}$] can regulate [ATP^{4-}] at levels appropriate for control of $^{86}Rb^+$ efflux. These considerations suggest that a primary role of the ecto-ATPase of J774 may be to limit the access of ATP^{4-} to its receptor, serving a function analogous to that of the acetylcholinesterase in cholinergic neurotransmission.

The variant J774 cell line ATPR B2 was at least 200-fold less sensitive to ATP-induced cation efflux but retained ecto-

ATPase activity with a V_{max} one-third of normal. This finding provides additional evidence that the ATP^{4-} receptor is distinct from the ecto-ATPase. Why these cells fail to respond to extracellular ATP is not clear; possible explanations include absence of the ATP^{4-} receptor, presence of a nonfunctional ATP^{4-} receptor, or alteration of a downstream event. Another possibility, that the ATP-resistant cells have a high level of ecto-ATPase activity that prevents access of ATP^{4-} to its receptor, has been eliminated by our finding that the ecto-ATPase activity of these cells is slightly lower than normal.

The physiologic role of secreted ATP is most firmly established in the nervous system, where ATP acts as a neurotransmitter in some nonadrenergic noncholinergic nerves (1). ATP is stored in granules in adrenal chromaffin cells, platelets, and neurons, but the function of this ATP is not understood. In the adrenal chromaffin granule, catecholamines and ATP are highly concentrated and form polymeric complexes which permit the chromaffin granules to remain osmotically stable (28). Chromaffin granules transport ATP as ATP^{4-} (29), and upon degranulation ATP^{4-} is released into the surrounding medium in high concentrations. Such a situation might also occur when ATP is released into a delimited space such as a synaptic cleft or an area of close apposition between a platelet and a macrophage. In these cases, the concentration of Mg^{2+} in this space or the ability of Mg^{2+} to diffuse into this space might be important factors in curtailing the interaction between ATP^{4-} and its receptor.

ATP may function as an intercellular messenger in nonneural as well as in neural systems and may be of particular importance in coordinating responses between different cells. The actions of platelet-secreted ATP are illustrative. Platelet aggregation releases ATP which in turn causes coronary artery vasodilation via interactions with endothelial cells (30). In addition, ATP secreted by platelets appears to cause vasodilation by a direct effect on the sympathetic nerves supplying the coronary artery (31). The vasodilatory effects of ATP are profound, and ATP is rapidly degraded in the blood, in part by the abundant ecto-ATPase on the lumenal surfaces of endothelial cells. Thus ATP storage, release, action, and degradation can be accomplished by cells of diverse lineage to regulate vascular tone.

While we know some of the effects of extracellular ATP on macrophages and the form of ATP required for these actions, we have little insight into the role of extracellular ATP in regulating macrophage activity *in vivo*. The dramatic changes in membrane permeability and resultant functional alterations observed in these cells in the presence of extracellular ATP suggest that the putative ATP^{4-} receptor may be important in regulating macrophage function within the vasculature and in tissues in response to release of ATP from platelets, endothelial cells, or neurons. In the case of neurons, ATP may form an important link in communications between the immune and nervous systems.

Acknowledgments—We thank Alan S. Newman for technical assistance and Dr. Joel A. Swanson for helpful discussion.

APPENDIX

To calculate $[ATP^{4-}]$ and $[MgATP^{2-}]$ for known $[Mg_{total}]$ and $[ATP_{total}]$ under the experimental conditions employed here, we have assumed that the predominant forms of ATP are $MgATP^{2-}$, $HATP^{3-}$, and ATP^{4-}. This approximation is reasonable because there was no extracellular Ca^{2+} present during these experiments, and other protonated and magnesium-containing chelates of ATP will be found in much lower concentration. Therefore, the relevant equilibria and conservation equations are as follows.

$$\frac{[MgATP^{2-}]}{[Mg^{2+}] \times [ATP^{4-}]} = K_{Mg} \quad (1)$$

$$\frac{[HATP^{3-}]}{[H^+] \times [ATP^{4-}]} = K_a \quad (2)$$

$$[Mg^{2+}] + [MgATP^{2-}] = [Mg_{total}] \quad (3)$$

$$[ATP^{4-}] + [MgATP^{2-}] + [HATP^{3-}] = [ATP_{total}] \quad (4)$$

By substituting for Mg^{2+}, $MgATP^{2-}$, and $HATP^{3-}$, one derives the following second degree equation for the only remaining variable, ATP^{4-}.

$$\{K_{Mg} + (K_{Mg})(K_a)([H^+])\} \times [ATP^{4-}]^2 + \{(K_{Mg})([Mg_{total}]) - (K_{Mg})([ATP_{total}]) + (K_a)([H^+]) + 1\} \times [ATP^{4-}] - [ATP_{total}] = 0$$

$[ATP_{total}]$, $[Mg_{total}]$, and $[H^+]$ are known; K_{Mg} and K_a were taken from Ref. 25. The quadratic equation can, therefore, be used to solve for $[ATP^{4-}]$, and $[MgATP^{2-}]$ can be determined from Equations 1 and 3.

REFERENCES

1. Burnstock, G. (1981) *J. Physiol.* **313**, 1–35
2. Burnstock, G. (1978) in *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach* (Straub, R. W., and Bolis, L., eds) pp. 107–118, Raven Press, New York
3. Krishtal, O. A., Marchenko, S. M., and Pidoplichko, V. I. (1983) *Neurosci. Lett.* **35**, 41–45
4. Douglas, W. W., Poisner, A. M., and Rubin, R. P. (1965) *J. Physiol.* **179**, 130–137
5. Meyers, K. M., Holmsen, H., and Seachord, C. L. (1982) *Am. J. Physiol.* **243**, R454–R461
6. Pearson, J. D., and Gordon, J. L. (1979) *Nature* **281**, 384–386
7. Charest, R., Blackmore, P. F., and Exton, J. H. (1985) *J. Biol. Chem.* **260**, 15789–15794
8. Cockcroft, S., and Gomperts, B. D. (1980) *Biochem. J.* **188**, 789–798
9. Becker, E. L., and Henson, P. M. (1975) *Inflammation* **1**, 71–84
10. Weisman, G. A., De, B. K., Friedberg, I., Pritchard, R. S., and Heppel, L. A. (1984) *J. Cell. Physiol.* **119**, 211–219
11. Chahwala, S. B., and Cantley, L. C. (1984) *J. Biol. Chem.* **259**, 13717–13722
12. Dubyak, G. R., and Young, M. B. D. (1985) *J. Biol. Chem.* **260**, 10653–10661
13. Maneryk, J. F., and Dryden, E. E. (1979) in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (Baer, H. P., and Drummond, G. I., eds) pp. 323–339, Raven Press, New York
14. DePierre, J. W., and Karnovsky, M. L. (1974) *J. Biol. Chem.* **249**, 7111–7120
15. Remold-O'Donnell, E. (1978) *J. Exp. Med.* **148**, 1099–1104
16. Ehrlich, Y. H., Davis, T. B., Bock, E., Kornecki, E., and Lenox, R. H. (1986) *Nature* **320**, 67–70
17. McCord, J. M., Petrone, W. F., and Jones, H. P. (1985) *Adv. Inflammation Res.* **10**, 21–29
18. Dahlquist, R., and Diamant, B. (1974) *Acta Pharmacol. Toxicol.* **34**, 368–384
19. Sung, S-S. J., Young, J. D-E., Origlio, A. M., Heiple, J. M., Kaback, H. R., and Silverstein, S. C. (1985) *J. Biol. Chem.* **260**, 13442–13449
20. Boyse, E. A., Old, L. J., and Chouroulinkor, J. (1964) *Methods Med. Res.* **10**, 39–47
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
22. Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics*, Butterworths, London
23. Edelson, P. J., and Erbs, C. (1978) *J. Exp. Med.* **147**, 77–86
24. Yount, R. G., Babcock, D., Ballantyne, W., and Ojala, D. (1971) *Biochemistry* **10**, 2484–2489
25. Alberty, R. A. (1968) *J. Biol. Chem.* **243**, 1337–1343
26. Michl, J., Ohlbaum, D. J., and Silverstein, S. C. (1976) *J. Exp. Med.* **144**, 1484–1493
27. Parker, J. C., and Snow, R. L. (1972) *Am. J. Physiol.* **223**, 888–893
28. Kopell, W. N., and Westhead, E. W. (1982) *J. Biol. Chem.* **257**, 5707–5710
29. Weber, A., and Winkler, H. (1981) *Neuroscience* **6**, 2269–2276
30. Houston, D. S., Shepherd, J. T., and Vanhoutte, P. M. (1985) *Am. J. Physiol.* **248**, H389–H395
31. Cohen, R. A. (1986) *J. Clin. Invest.* **77**, 369–375

Exhibit B

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 266, No. 17, Issue of June 15, pp. 10796-10801, 1991
Printed in U.S.A.

Reduced High Density Lipoprotein Cholesterol in Human Cholesteryl Ester Transfer Protein Transgenic Mice*

(Received for publication, November 9, 1990)

Luis B. Agellon‡, Annemarie Walsh§, Tony Hayek§, Philippe Moulin¶, Xian Cheng Jiang, Samuel A. Shelanski, Jan L. Breslow§, and Alan R. Tall

From the Division of Molecular Medicine, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032 and the §Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University, New York, New York 10021

The human cholesteryl ester transfer protein (CETP) facilitates the exchange of neutral lipids among lipoproteins. In order to evaluate the effects of increased plasma CETP on lipoprotein levels, a human CETP minigene was placed under the control of the mouse metallothionein-I promoter and used to develop transgenic mice. Integration of the human CETP transgene into the mouse genome resulted in the production of active plasma CETP. Zinc induction of CETP transgene expression caused depression of serum cholesterol due to a significant reduction of high density lipoprotein cholesterol. There was no change in total cholesterol content in very low and low density lipoproteins. However, there was a decrease in the free cholesterol/cholesteryl ester ratio in plasma and in all lipoprotein fractions of transgenic mouse plasma, suggesting stimulation of plasma cholesterol esterification. The results suggest that high levels of plasma CETP activity may be a cause of reduced high density lipoproteins in humans.

HDL[1] plays an important role in the transport of plasma cholesterol (1, 2). This particle is thought to participate in the process of reverse cholesterol transport by which excess cholesterol from peripheral tissues is returned to the liver for use or excretion. There is a strong inverse relationship between plasma HDL cholesterol concentration and incidence of atherosclerosis. Thus, factors that influence HDL cholesterol metabolism may have special significance to atherogenesis. One of these is plasma CETP, a hydrophobic single chain glycoprotein of M_r 74,000 (3, 4). Its action results in the net transfer of CE from HDL to VLDL and LDL (2). Plasma CETP levels in normal (5) and dyslipidemic[2] humans range from about 1 to 8 μg/ml, but it is not known if increased concentrations of CETP can result in decreased HDL cholesterol levels. In this report, we describe the development of transgenic mice that express human plasma CETP. The mouse is especially suited for these studies since this species normally lacks plasma CE transfer activity (6). Our findings indicate that high levels of plasma CETP activity *in vivo* can cause lower HDL cholesterol levels.

EXPERIMENTAL PROCEDURES

Development of CETP Transgenic Mice—A 6.3-kilobase pair synthetic CETP structural gene was assembled by combining genomic and cDNA fragments. The 5′-genomic region (including 138 base pairs of flanking sequence, exon 1, intron 1, and part of exon 2) and the 3′-genomic region (including part of exon 13, exons 14–16, introns 13–15, and 121 base pairs of flanking sequence) were linked together using a fragment taken from the human CETP cDNA. This fusion resulted in the complete removal of introns 2–12 and generation of one synthetic exon. No alterations were made to the remaining CETP sequence. A fully assembled CETP transgene was obtained by linking the CETP minigene to the mouse metallothionein-I promoter (7). To generate transgenic mice, the CETP transgene (vector sequences removed) was microinjected into the male pronuclei of fertilized mouse eggs taken from superovulated (C57BL/6J × CBA/J)F_1 females. Injected embryos were implanted into the oviducts of surrogate females of the same genetic background (8).

To simplify the identification of transgenic mice, DNA from tail tips of 2–3-week-old mice were screened for human CETP gene sequences by polymerase chain reaction amplification (9). Primers were directed to amplify the last two exons of the human CETP gene. Amplification reactions using nontransgenic mouse DNA as template yielded no amplification products. DNA of mice that tested positive by polymerase chain reaction amplification were characterized further by DNA blotting using the human CETP cDNA as a probe. The founder mouse was mated with (C57BL/6J × CBA/J) mice to establish a line.

RNA Analysis—RNA was extracted from tissues of mice (both sexes, transgenic and nontransgenic), given chow and distilled water (basal) or 25 mM $ZnSO_4$ for 7 days (Zn-induced). Total RNA (30 μg) from the tissues was analyzed for CETP mRNA by a solution hybridization-ribonuclease protection assay (10) using a riboprobe highly specific for the human CETP mRNA. The riboprobe (250 nucleotides) contains a portion of the vector (Bluescript KS+), and is complementary to the last 28 codons plus 58 nucleotides of the 3′-untranslated region of the human CETP mRNA.

Measurement of Plasma CETP Concentration and Activity—The amount of CETP in mouse plasma was measured by solid phase radioimmunoassay (5). CETP activity was measured as described previously (11) with slight modifications. Plasma (2.5 μl) was added to the assay mixture containing HDL (20 μg total of cholesterol, 20,000 cpm), LDL (0.4 μg of protein), α_2-macroglobulin (80 μg), and diethyl-*p*-nitrophenylphosphate (1 mM). The mixture (132.5 μl) was incubated at 37°C overnight. The assay was linear from 1.25 to 5 ng of CETP.

Diets—Heterozygote transgenic mice from the F_2 generation were used to determine the effects of CETP expression on the mouse lipoprotein pattern. The mice (transgenic and nontransgenic littermates) were housed in metabolic cages and given free access to food and water. Mice were first given a chow diet (Purina laboratory

* This research was supported by National Institutes of Health Grant HL43165 and HL22682 (to A. R. T.) and Grants HL32435 and HL33714 (to J. L. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Medicine, Columbia University College of Physicians and Surgeons, 630 W. 168th St., New York, NY 10032

¶ Fellow supported by a grant from the Institut National de la Santé et de la Recherche Médicale (France).

[1] The abbreviations used are: HDL, high density lipoprotein; CETP, cholesteryl ester transfer protein; FC, free cholesterol; CE, cholesteryl ester; VLDL, very low density lipoprotein; LDL, low density lipoprotein; apo, apolipoprotein.

[2] P. Moulin and A. R. Tall, unpublished observations.

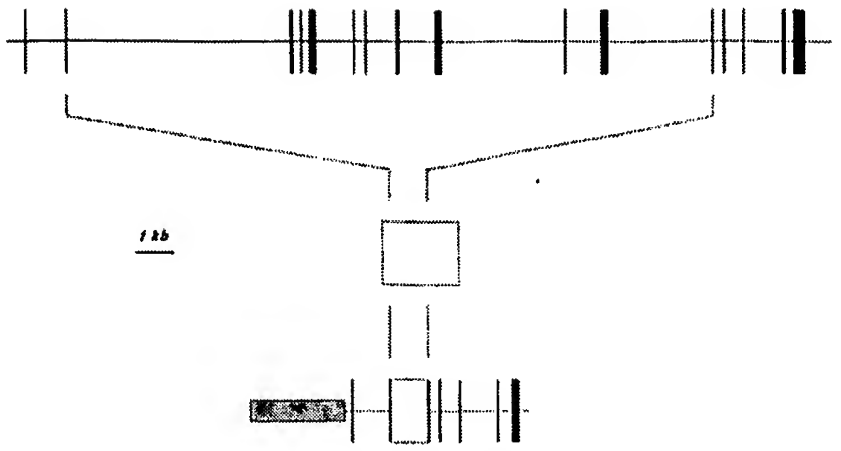

FIG. 1. **Structure of the CETP transgene.** The organization of the human CETP gene is shown (*top*). The *vertical bars* represent exons. A minigene derivative was constructed by combining genomic and cDNA fragments (*middle*). The fusion of these fragments produced one synthetic exon (*open box*, *bottom*). The fully assembled transgene (*bottom*) was placed under the control of the mouse metallothionein-I promoter (*hatched box*) and then used to develop transgenic mice. *kb*, kilobase pairs.

rodent chow 5001 (diet 1)) and distilled water for 7 days and then given a chow diet and water supplemented with 10 mM $ZnSO_4$ for 3 days to raise the level of plasma CETP. Subsequently, the same animals were given a high fat (19% by weight) diet (formulation TD 88333, Teklad Premier (diet 2)) and distilled water for 7 days and then the high fat diet and water supplemented with 25 mM $ZnSO_4$ for an additional 7 days. Prior to each dietary change, mice were fasted overnight, and blood was collected the following morning from the retroorbital plexus under methoxyflurane anesthesia. In further experiments, pooled plasma from mice given a diet containing 7.5% (w/w) fat, 1.25% (w/w) cholesterol, 0.5% (w/w) sodium cholate (formulation TD 90221, Teklad Premier (diet 3)), and water supplemented with 25 mM $ZnSO_4$ was used.

Plasma Lipid and Lipoprotein Analyses—For small volumes of mouse plasma, HDL was separated from apolipoprotein (apo) B-containing lipoproteins by dextran sulfate precipitation. Using this method, an insignificant amount of mouse apoA-I (measured by rocket immunoelectrophoresis) is precipitated.[3] Other lipoproteins were isolated by ultracentrifugation in an Airfuge (Beckman Instruments) exactly as described by Walsh *et al.* (8). For large scale separation, lipoproteins (VLDL, $d < 1.006$ g/ml; LDL, $d = 1.006$–1.063 g/ml; and HDL, $d = 1.063$–1.21 g/ml) from 1 ml of pooled transgenic or nontransgenic mouse plasma were isolated by sequential density ultracentrifugation. Total cholesterol and FC levels were determined by enzymatic methods using commercial kits (Boehringer Mannheim and Sigma).

To estimate lipoprotein size, plasma lipoproteins were stained with Sudan Black prior to electrophoresis of plasma on native 4–30% polyacrylamide gradient gels (Pharmacia LKB Biotechnology Inc.) (12). The mobility of isolated lipoprotein fractions on agarose gels (Titan gel lipoprotein system, Helena Laboratories) was visualized by staining with Fat Red 7B.

RESULTS

The human CETP cDNA and gene have recently been cloned and characterized (13, 14). Since the CETP gene is large and since transgenes based on cDNAs are often poorly expressed (15), we constructed a minigene version of the human CETP gene (Fig. 1) to facilitate the development of transgenic mice. Out of 70 mice born from injected fertilized mouse eggs, one male and one female were found to have integrated the transgene. A transgenic line was established from the female founder. The male founder died before a line could be established. Southern blotting of the DNA isolated from the female founder and from subsequent generations indicated that only one copy of the transgene had integrated (not shown).

To determine if CETP transgenic mice expressed CETP, plasma was screened for CETP immunologically and for lipid transfer activity. Plasma was passed through an immunoaffinity column constructed with a monoclonal antibody (mAb TP2), which recognizes an epitope at the carboxyl terminus of CETP (16) and inhibits CETP activity (17). Immunoblots of immunoretained column fractions revealed the existence of a mAb TP2 immunoreactive protein in transgenic mouse plasma (Fig. 2, *lanes 1* and 2), whereas none was detected in the plasma of nontransgenic littermates (Fig. 2, *lane 3*). The size of the CETP in mouse plasma was identical with that found in human plasma (3), and activity could be inhibited by TP2; no CETP mass nor CETP activity was detected in the plasma of nontransgenic mice. Together, these results show that the CETP transgene is expressed and correctly encodes the carboxyl-terminal TP2 epitope, and that the protein produced is properly processed and functionally active.

RNA from various tissues were analyzed for CETP mRNA by solution hybridization-ribonuclease protection using a probe specific for human CETP mRNA. Under uninduced (basal) conditions, moderate amounts of the CETP mRNA were detected in adipose tissue, heart, and brain; smaller amounts were found in liver, small intestine, and muscle (Fig. 3). Interestingly, the pattern of expression and the relative abundance of the CETP mRNA among the mouse tissues resemble those found in humans (18). The CETP minigene carries small portions of the 5′- and 3′-flanking regions, as well as five of the introns of the natural CETP gene. These regions may contain regulatory elements that influence the tissue-specific expression of the CETP transgene.

Addition of zinc to the drinking water caused an increase of CETP mRNA in the small intestine and liver (Fig. 3), indicating that the expression of the transgene can be induced by heavy metals. Considering the relative sizes of the organs and their CETP mRNA abundance, the liver is likely to constitute the major site of CETP synthesis in the induced state.

Under basal conditions, the level of CETP in the plasma of heterozygote transgenic mice ranged from 1.6 to 2.3 μg/ml (Table I), similar to that in normal human plasma (5). Among transgenic mice, the females tended to have higher plasma CETP levels than males (1.35× on average), confirming the results obtained by immunoblotting (Fig. 2). The basis for this difference is not clear but may be related to the sex differences in lipid metabolism or to differential expression of the transgene by the two sexes (see Fig. 2). The initial zinc treatment (10 mM $ZnSO_4$), which was given for 3 days, resulted in a modest increase (to 140%) of plasma CETP levels

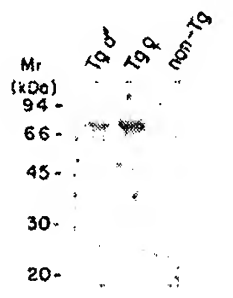

FIG. 2. **Detection of human CETP in transgenic mouse plasma.** Mouse plasma was passed through a CETP immunoaffinity column constructed with a monoclonal antibody (mAb TP2) that recognizes an epitope at the carboxyl terminus of human CETP (16). The retained fraction was eluted, blotted, and then probed with ^{125}I-TP2. An immunoreactive protein is clearly visible in the plasma of CETP transgenic mice (*lane 1*, pooled female plasma; *lane 2*, pooled female plasma). Pooled plasma from nontransgenic littermates contains no detectable TP2 immunoreactive protein (*lane 3*). Mobility of molecular weight standards are indicated on the *left side* of the figure. *Tg*, transgenic.

[3] T. Hayek, Y. Ito, and J. L. Breslow, unpublished observations.

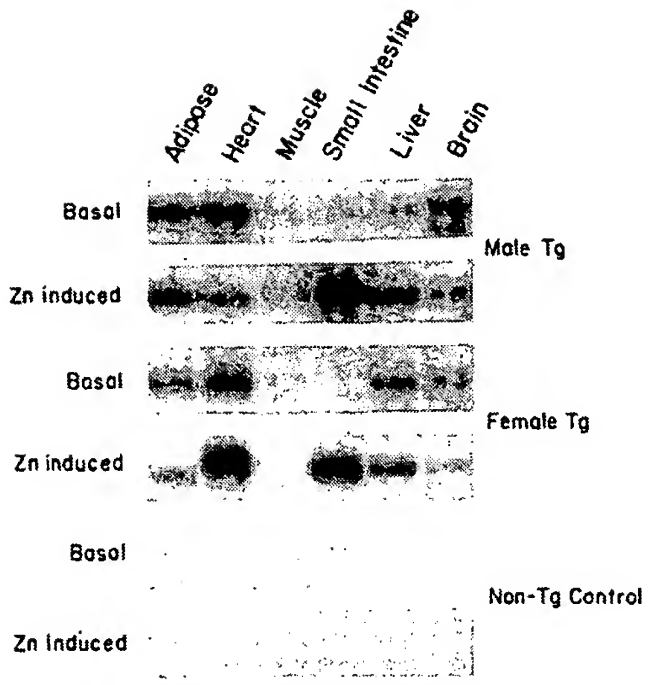

FIG. 3. **Sites of CETP transgene expression.** CETP mRNA was detected in mouse tissues as described under "Experimental Procedures." A protected fragment (140 nucleotides) is detected in RNA extracted from transgenic mouse tissues (*top four panels*) but not in RNA extracted from nontransgenic mouse tissues (*bottom two panels*). *Basal,* RNA prepared from tissues of mice given distilled water; *Zn induced,* RNA prepared from tissues of mice given 25 mM $ZnSO_4$ for 7 days; *Tg,* transgenic.

(Table I). The second zinc treatment, which was given at a higher dose (25 mM $ZnSO_4$) and for a longer period (7 days), resulted in an even greater increase (to 200%) of plasma CETP levels.

We compared the lipoprotein profiles of transgenic and nontransgenic mice on both chow and high fat diets (diets 1 and 2, respectively) to determine whether human CETP has any impact on the mouse lipoprotein pattern. On both diets 1 and 2, zinc induction caused a significant fall in total plasma cholesterol due to a marked decrease in plasma HDL cholesterol concentration (Table II). On diet 1, HDL levels of zinc-treated male and female transgenic mice fell by 27 and 15%, respectively; on diet 2, HDL levels of zinc-treated male and female transgenic mice fell by 25 and 28%, respectively. Zinc-treated transgenic mice on both diets 1 and 2 had lower HDL cholesterol levels than the nontransgenic controls studied under the same conditions. Compared to nontransgenic controls, HDL levels of zinc-treated transgenic males and females on diet 1 were 12 and 17% lower, respectively; on diet 2, the HDL levels of zinc-treated transgenic males and females were 27 and 38% lower, respectively. No significant changes in total cholesterol content of VLDL + LDL fractions (Table II), or separated VLDL and LDL fractions, were observed. In addition, no significant differences in lipoprotein triglyceride concentrations were observed between transgenic and nontransgenic mice. Similar results were obtained when zinc-treated mice were maintained on diet 3. HDL cholesterol levels of transgenic mice were 37% lower compared to the nontransgenic controls (Table III). These results indicate that the major effect of high plasma CETP activity is the marked reduction of HDL cholesterol levels.

FC and CE composition analysis of transgenic and nontransgenic mouse plasma (diet 3) revealed a significant decrease in the FC/CE ratio in transgenic mice (77% of the value in nontransgenic controls, $n = 5$, $p = 0.04$). Analysis of lipoproteins, fractionated from pooled plasma by preparative density ultracentrifugation, showed the decrease of FC/CE ratios in all lipoprotein fractions, with the largest change in the VLDL fraction (Table III). The decrease in ratio was seen in all lipoprotein fractions, suggesting that lecithin:cholesterol acyltransferase activity may be stimulated in CETP transgenic mice.

The changes in the plasma HDL cholesterol concentration of transgenic mice were independent of apoA-I levels, since there were no significant differences in plasma apoA-I concentrations before and after zinc treatment (transgenic males, 132 *versus* 134% of control;[4] transgenic females, 89 *versus* 89% of control). This suggests that the reduction of HDL cholesterol levels should be accompanied by a reduction of HDL particle size. This was confirmed by native polyacrylamide gradient gel electrophoresis of Sudan Black-stained plasma (Fig. 4). In both sexes, zinc induction of CETP transgene expression led to a reduction of HDL particle size (in pooled plasma) from 9.7 to 9.3 nm in males (Fig. 4, *lanes 2* and *3*), and from 10.2 to 9.9 nm in females (Fig. 4, *lanes 4* and *5*). To rule out the possibility that this was the effect of zinc rather than CETP levels, a gene dosage experiment was carried out. Plasma CETP activity in the homozygotes was 1.6-fold greater than in heterozygotes. Progressive increases in gene dosage, from nontransgenic (Fig. 4, *lane 6*) to heterozygote (Fig. 4, *lane 7*) to homozygote (Fig. 4, *lane 8*) states led to progressive reductions in HDL particle size from 10.3 to 10.0 to 9.5 nm, respectively. These results confirm that CETP transgene expression causes a decrease in HDL particle size.

The gradient gels also show an increase in the intensity of LDL lipid staining in the pooled plasma of zinc-treated transgenic mice on both diet 2 (Fig. 4; males, *lanes 2* and *3*; females, *lanes 4* and *5*) and diet 3 (not shown). These effects were not evident in nontransgenic mice treated with zinc. Increased LDL lipid staining was also evident in the plasma of heterozygote and homozygote transgenic mice in the absence of zinc induction (Fig. 4, *lanes 7* and *8*). These results appear to be in conflict with the lack of significant difference of total LDL cholesterol of transgenic and nontransgenic mice. One explanation for the increased lipid staining of LDL is an increased content of CE (Table III); CE, particularly those enriched in polyunsaturated fatty acids, have high avidity for Sudan Black (19).

Since both HDL_1 and LDL are present in the $d = 1.006$–1.063 g/ml density range (20), we also considered the possibility that there might be CE transfer from HDL_1 to LDL, which could result in no net change of cholesterol content in this density fraction. To assess this possibility, mouse lipoproteins were isolated by sequential ultracentrifugation and analyzed by agarose gel electrophoresis (Fig. 5). The HDL_1 was visualized as a minor component (Fig. 5, *arrow*) in the LDL density fraction of nontransgenic mouse plasma, as expected for this strain of mice (21). In addition, the amount of apoA-I in the LDL density fraction of nontransgenic mice was less than 2% of the total in plasma. HDL_1 is absent in CETP transgenic mice (Fig. 5), showing that this lipoprotein is an excellent substrate for CETP, as suggested in earlier studies (22–24). Thus, we conclude that the increased lipid staining of transgenic mouse LDL (Fig. 4) is largely due to a change in lipid composition (*i.e.* decrease in FC/CE ratio) but may also reflect a minor transfer of CE from HDL_1 to LDL in the $d = 1.006$–1.063 g/ml density range.

DISCUSSION

The effect of reduced CETP activity on HDL levels has been demonstrated in several studies. In rabbits, administration of monoclonal antibodies that block CETP activity

[4] Pooled normal mouse plasma.

TABLE I

Plasma CETP concentration and activity of CETP transgenic mice under different dietary conditions

CETP concentration and activity were determined as described under "Experimental Procedures." The reference human plasma has 2.4 ng/μl CETP and transfer activity of 463 cpm/μl under the described assay conditions. The calculated specific activity of CETP in human plasma is 193 cpm/ng. Differences in CETP activity between treatments were evaluated using Student's *t* test. Values shown are mean ± S.D. ($n = 6$ per group, except for the nontransgenic female group during the chow diet period, where $n = 5$). Differences were considered significant if $p < 0.05$. Values for CETP concentration are from pooled transgenic mouse plasma and represent the means of two determinations.

CETP	Chow diet				High fat diet			
	Basal		Zn-induced		Basal		Zn-induced	
	Males	Females	Males	Females	Males	Females	Males	Females
Activity (cpm/μl)	573 ± 71	585 ± 170	917 ± 140[a]	860 ± 244[b]	611 ± 169	704 ± 186	1058 ± 125[c]	1045 ± 99[d]
Concentration (ng/μl)	1.6	2.3	2.3	3.6	1.7	1.9	3.5	4.2
Specific activity (cpm/ng)	354	231	394	239	357	369	302	247

[a] Different from basal, $p < 0.0001$.
[b] Different from basal, $p = 0.011$.
[c] Different from basal, $p < 0.0001$.
[d] Different from basal, $p = 0.006$.

TABLE II

Lipoprotein profiles of nontransgenic and CETP transgenic mice under different dietary conditions

See "Experimental Procedures" for details concerning diets, plasma and lipoprotein preparations, and lipid analyses. Values shown are mean ± S.D. ($n = 6$ per group, except for the nontransgenic female group during the chow diet period, where $n = 5$). Differences in HDL cholesterol concentration between groups and between treatments were evaluated using Student's *t* test and one-way analysis of variance (ANOVA). Differences were considered significant if $p < 0.05$.

Mice	Chow diet						High fat diet					
	Basal			Zn-induced			Basal			Zn-induced		
	Total	VLDL + LDL	HDL	Total	VLDL + LDL	HDL	Total	VLDL + LDL	HDL	Total	VLDL + LDL	HDL
	mg/dl of cholesterol											
Males												
Nontransgenic	73 ± 4	21 ± 3	53 ± 4	74 ± 5	24 ± 3	50 ± 2	132 ± 19	35 ± 7	97 ± 14	135 ± 19	38 ± 9	97 ± 16
Transgenic	80 ± 7	20 ± 4	60 ± 7	66 ± 3	22 ± 1	44 ± 2[a]	128 ± 21	33 ± 6	95 ± 15	108 ± 13	36 ± 8	71 ± 9[b]
Females												
Nontransgenic	63±10	17±2	45±8	57±11	16 ± 5	41 ± 6	110 ± 14	32 ± 7	78 ± 10	122 ± 14	43 ± 12	79 ± 10
Transgenic	60±5	20±2	40±4	51±4	17 ± 3	34 ± 4[c]	94 ± 8	26 ± 3	68 ± 7	79 ± 10	30 ± 5	49 ± 8[d]

[a] Different from the basal state (*t* test, $p < 0.001$; one-way ANOVA, $p < 0.005$); different from nontransgenic males (*t* test, $p < 0.01$; one-way ANOVA, $p < 0.03$).
[b] Different from the basal state (*t* test, $p < 0.03$; one-way ANOVA, $p < 0.01$); different from nontransgenic males (*t* test, $p < 0.005$; one-way ANOVA, $p < 0.001$).
[c] Different from the basal state (*t* test, $p < 0.02$; one-way ANOVA, $p < 0.05$); different from nontransgenic females (one-way ANOVA, $p = 0.011$).
[d] Different from the basal state (*t* test, $p < 0.001$; one-way ANOVA, $p < 0.005$); different from nontransgenic females (*t* test, $p < 0.001$; one-way ANOVA, $p < 0.001$).

caused an elevation of HDL levels (25). Humans with CETP deficiency, caused by a CETP gene mutation, have markedly elevated HDL levels (24, 26). Although these studies clearly show that reduced plasma CETP levels cause increased HDL concentrations, the effects of increased CETP activity on plasma HDL levels are largely unknown. In several clinical conditions, such as Type III hyperlipidemia, nephrotic syndrome, and patients taking probucol (27), a high CETP level is associated with low HDL cholesterol levels. However, it is not known whether the inverse relationship of CETP and HDL levels is causal. Past experiments designed to explore the direct effects of high plasma CETP on lipoprotein profiles employed infusion of human CETP into rats. However, the administered fraction was impure, and the dose was uncontrolled (22). In these experiments, significant reductions in HDL cholesterol were observed only under certain conditions (28).

Transgenic mice have been used to model human lipoprotein metabolism in several studies. In these mice, inappropriate production of certain proteins involved in lipid metabolism has been shown to cause dyslipidemia. For example, overproduction of human apoA-I in mice results in hyperalphalipoproteinemia (8, 29); overexpression of human apoC-III results in hypertriglyceridemia (30); and overproduction of the human LDL receptor causes rapid clearance of LDL from plasma (31). These earlier studies prompted us to evaluate the effects of CETP expression in mice, which normally lack CE transfer activity in plasma.

The present study demonstrates that high levels of plasma CETP in transgenic mice cause reduced HDL levels, and suggest the possibility that elevated plasma CETP concentration might sometimes be causally related to hypoalphalipoproteinemia in humans. Surprisingly, the reduction did not affect plasma apoA-I concentrations, thus resulting in a decrease of HDL particle size. This is compatible with the results obtained in families with CETP deficiency, where CETP levels have a much larger effect on HDL cholesterol than apoA-I levels (24). CETP in the mouse did not have a major quantitative effect on VLDL or LDL cholesterol. However, the lipid composition of these fractions was altered, shown by a decrease in FC/CE ratio and increased lipid staining. An increase in the CE content of LDL, along with the expected

TABLE III

Total cholesterol, free cholesterol, and cholesteryl ester content of plasma and isolated lipoproteins from plasma of zinc-treated mice maintained on a high fat/high cholesterol diet

Preparation of lipoprotein fractions from small volumes of mouse plasma and determination of cholesterol content were done as described under "Experimental Procedures." Values shown for cholesterol are mean ± S.D. ($n = 5$ for transgenic mice, $n = 6$ for nontransgenic mice). Total and free cholesterol contents of mouse lipoproteins (VLDL, $d < 1.006$ g/ml; LDL, $d = 1.006$–1.063 g/ml; HDL, $d = 1.063$–1.21 g/ml) isolated by sequential density ultracentrifugation are also shown. FC/CE mass ratios for VLDL, LDL, and HDL (means of duplicate assays) are from pooled mouse plasma ($n = 5$ for transgenic, $n = 6$ for nontransgenic). Differences in cholesterol content between transgenic and nontransgenic mouse lipoproteins were evaluated using Student's t test and one-way analysis of variance. Differences were considered significant if $p < 0.05$.

Mice	VLDL	LDL	HDL
Total cholesterol (mg/dl)			
Nontransgenic	73 ± 16	44 ± 10	73 ± 15
Transgenic	68 ± 21	49 ± 18	46 ± 10[a]
FC/CE mass ratio			
Nontransgenic	0.32	0.44	0.38
Transgenic	0.15	0.31	0.32

[a] Different from nontransgenic mice (t test, $p < 0.005$; one-way ANOVA, $p < 0.01$).

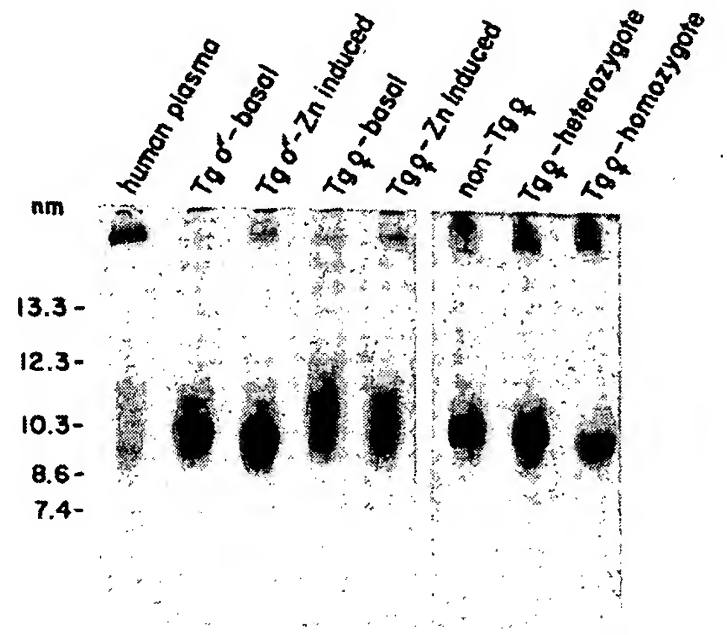

FIG. 4. **Native gradient gel electrophoresis of transgenic mouse plasma.** Plasma (25-μl) samples were stained with Sudan Black prior to electrophoresis on a native 4–30% polyacrylamide gradient gel. The figure shows lipid-stained lipoproteins in human plasma (*lane 1*), in pooled plasma ($n = 5$) from heterozygote CETP transgenic male and female mice (*lanes 2* and *4*, respectively), from heterozygote CETP transgenic male and female mice treated with zinc (*lanes 3* and *5*, respectively), and in plasma of a nontransgenic female mouse, a heterozygous CETP transgenic female mouse, and a homozygous CETP transgenic female mouse (*lanes 6–8*, respectively). The broad HDL migration pattern of heterozygous transgenic female mice (*lanes 4* and *5*) likely reflects the heterogeneous genetic background of this generation. These mice were derived from a transgenic (C57BL/6 × CBA) × nontransgenic (C57BL/6 × CBA) cross. The samples shown in *lanes 6–8* are from individual female mice. These mice were obtained from a sibling cross and have more limited genetic variability. The hydrodynamic diameters (*nm*) estimated from the mobility of protein standards are indicated on the *left side* of the figure. *Tg*, transgenic.

increase of polyunsaturated fatty acids in CE (32) might increase the susceptibility of LDL to oxidative modification, rendering the LDL more atherogenic.

In all lipoprotein fractions of transgenic mouse plasma, the FC/CE ratio was decreased. The increased CE content suggests an increase in lecithin:cholesterol acyltransferase activity, perhaps to replace the CE transferred out of HDL into apoB-containing lipoproteins. Previous *in vitro* studies looking at the effect of CETP on lecithin:cholesterol acyltransferase activity gave conflicting results. Inhibition of CETP activity with a monoclonal antibody did not affect the cholesterol esterification rate of human plasma (33). However, another study, using synthetic substrates, suggested that CETP may stimulate lecithin:cholesterol acyltransferase activity (34). The optimal substrate for lecithin:cholesterol acyltransferase is nascent HDL. In transgenic mice, the CETP produced in tissues may effect the removal of lecithin:cholesterol acyltransferase product from HDL and allow an apparent increase in lecithin:cholesterol acyltransferase activity.

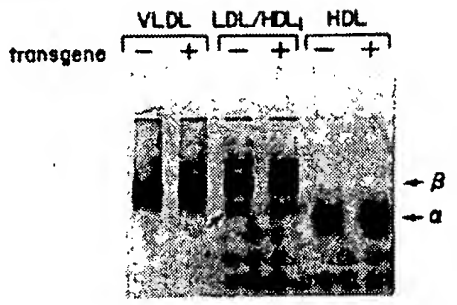

FIG. 5. **Agarose gel electrophoresis of mouse plasma.** Lipoproteins were isolated from pooled nontransgenic (−) or transgenic mouse (+) plasma fed a high fat/high cholesterol diet and given water supplemented with 25 mM zinc sulfate. Mobility of lipoproteins on agarose gels was visualized by staining with Fat Red 7B. The *arrow* shows lipoproteins with α-mobility (HDL_1) in the $d = 1.006$–1.063 g/ml density range of nontransgenic mouse plasma.

No significant differences in HDL cholesterol levels were apparent between transgenic and nontransgenic mice in the uninduced state, despite the presence of considerable CETP mass in the plasma. Effective CETP activity might be influenced by a number of factors. The existence of a protein inhibitor in rodent plasma has long been suspected (35). However, our activity measurements revealed that the human CETP was actually somewhat more active in mouse plasma than in human plasma, and there were no significant changes in specific activity after zinc treatment (see Table I). A second possibility may be related to the site of CETP transgene expression. In the basal state, CETP expression in the liver is low, whereas it increases dramatically after zinc treatment (see Fig. 2). High level expression of CETP in the liver may have more impact on HDL levels, due to the co-ordinated action of CETP and hepatic lipase that could enhance the transfer of CE out of HDL (36). Another possibility is that the bulk of mouse plasma HDL is not an optimal substrate for CETP.[5] As noted in the present and in earlier studies (22–24), larger apoA-I-containing HDL species (HDL_1 or HDL_{2b}) are the optimal substrates for CETP, but these particles are relatively minor components of HDL in the CETP transgenic mice (see Figs. 4 and 5). This represents a limitation of the present model but could be overcome by crossing CETP transgenic mice with human apoA-I transgenic mice, which have markedly increased levels of human apoA-I-containing HDL_2 particles (29).

An unexpected finding of the present study was that the pattern of expression and the relative abundance of the CETP mRNA in uninduced CETP transgenic mice resemble that found in humans (18). This is in contrast with some human apolipoprotein transgenes that show loss of tissue-specific expression in transgenic mice (8, 29, 37, 38). However, in the

[5] Immunoblotting of plasma obtained from transgenic mice under the basal state and separated on gradient gels indicated that ~30% of CETP is bound to mouse HDL; the remaining fraction is free.

case of the human apoE gene, the correct tissue-specific pattern of expression in transgenic mice can be restored when large amounts of flanking DNA are incorporated into the transgene (37). Thus, it appears that the CETP minigene, which has minimal amounts of the 5′- and 3′-flanking regions and only some of its introns (see Fig. 1), contains regulatory elements that can influence the tissue-specific expression of the CETP transgene. This suggestion will need to be confirmed in transgenic mice that carry the human CETP minigene linked to its natural flanking regions and their derivatives.

REFERENCES

1. Gordon, D. J., and Rifkind, B. M. (1989) *N. Engl. J. Med.* **321**, 1311–1316
2. Tall, A. R. (1990) *J. Clin. Invest.* **86**, 379–384
3. Hesler, C. B., Swenson, T. L., and Tall, A. R. (1987) *J. Biol. Chem.* **262**, 2275–2282
4. Jarnagin, A. S., Kohr, W., and Fielding, C. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 1854–1857
5. Marcel, Y. L., McPherson, R., Hogue, M., Czarnecka, H., Zawadzki, Z., Weech, P. K., Whitlock, M. E., Tall, A. R., and Milne, R. W. (1990) *J. Clin. Invest.* **85**, 10–17
6. Jiao, S., Cole, T. G., Kitchens, T. T., Pfleger, B., and Schonfeld, G. (1990) *Metabolism* **39**, 155–160
7. Palmiter, R. D., Norstedt, G., Gelinas, R. E., Hammer, R. E., and Brinster, R. L. (1983) *Science* **222**, 809–814
8. Walsh, A., Ito, Y., and Breslow, J. L. (1989) *J. Biol. Chem.* **264**, 6488–6494
9. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) *Science* **239**, 487–491
10. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., and Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035–7056
11. Tall, A. R., Granot, E., Brocia, R., Tabas, I., Hesler, C., Williams, K., and Denke, M. (1987) *J. Clin. Invest.* **79**, 1217–1225
12. LeBoeuf, R. C., Doolittle, M. H., Montcalm, A., Martin, D. C., Reue, K., and Lusis, A. J. (1990) *J. Lipid Res.* **31**, 91–101
13. Drayna, D., Jarnagin, A. S., McLean, J., Henzel, W., Kohr, W., Fielding, C., and Lawn, R. (1987) *Nature* **237**, 632–634
14. Agellon, L. B., Quinet, E. M., Gillette, T. G., Drayna, D. T., Brown, M. L., and Tall, A. R. (1990) *Biochemistry* **29**, 1372–1376
15. Brinster, R. L., Allen, J. M., Behringer, R. R., Gelinas, R. E., and Palmiter, R. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 836–840
16. Swenson, T. L., Hesler, C. B., Brown, M. L., Quinet, E., Trotta, P. P., Haslanger, M. F., Gaeta, F. C. A., Marcel, Y. L., Milne, R. W., and Tall, A. R. (1989) *J. Biol. Chem.* **264**, 14318–14326
17. Hesler, C. B., Tall, A. R., Swenson, T. L., Weech, P. K., Marcel, Y. L., and Milne, R. W. (1988) *J. Biol. Chem.* **263**, 5020–5023
18. Jiang, X. C., Moulin, P., Quinet, E., Goldberg, I. J., Yacoub, L. K., Agellon, L. B., Compton, D., Schnitzer-Polokoff, R., and Tall, A. R. (1991) *J. Biol. Chem.* **266**, 4631–4639
19. Hatch, F. T., and Lees, R. S. (1968) *Adv. Lipid Res.* **6**, 1–68
20. Weisgraber, K. H., and Mahley, R. W. (1983) in *CRC Handbook of Electrophoresis* (Lewis, L. A., and Naito, H. K., eds) pp. 103–132, CRC Press, Boca Raton
21. Breckenridge, W. C., Roberts, A., and Kuksis, A. (1985) *Arteriosclerosis* **5**, 256–264
22. Ha, Y. C., Chiang, L. B. F., and Barter, P. J. (1985) *Biochim. Biophys. Acta* **833**, 203–210
23. Yamashita, S., Sprecher, D. L., Sakai, N., Matsuzawa, Y., Tarui, S., and Hui, D. Y. (1990) *J. Clin. Invest.* **86**, 688–695
24. Inazu, A., Brown, M. L., Hesler, C. B., Agellon, L. B., Koizumi, J., Takata, K., Maruhama, Y., Mabuchi, H., and Tall, A. R. (1990) *N. Engl. J. Med.* **323**, 1234–1238
25. Whitlock, M. E., Swenson, T. L., Ramakrishman, R., Leonard, M. T., Marcel, Y. L., Milne, R. W., and Tall, A. R. (1989) *J. Clin. Invest.* **84**, 129–137
26. Brown, M. L., Inazu, A., Hesler, C. B., Agellon, L. B., Mann, C., Whitlock, M. E., Marcel, Y. L., Milne, R. W., Koizumi, J., Mabuchi, H., Takeda, R., and Tall, A. R. (1989) *Nature* **342**, 448–451
27. McPherson, R., Hogue, M., Milne, R. W., Tall, A. R., and Marcel, Y. L. (1991) *Arteriosclerosis*, in press
28. Ha, Y. C., and Barter, P. J. (1986) *Comp. Biochem. Physiol.* **83B**, 462–466
29. Rubin, E. M., Ishida, B. Y., Clift, S. M., and Krauss, R. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 434–438
30. Ito, Y., Azrolan, N., O'Connell, A., Walsh, A., and Breslow, J. L. (1990) *Science* **249**, 790–793
31. Hofman, S. L., Russell, D. W., Brown, M. S., Goldstein, J. L., and Hammer, R. E. (1988) *Science* **239**, 1277–1281
32. Bisgaier, C. L., Siebenkas, M. V., Brown, M. L., Inazu, A., Koizumi, J., Mabuchi, H., and Tall, A. R. (1991) *J. Lipid Res.* **32**, 21–44
33. Yen, F. T., Deckelbaum, R. J., Mann, C. J., Marcel, Y. L., Milne, R. W., and Tall, A. R. (1989) *J. Clin. Invest.* **83**, 2018–2024
34. Chajek, T., Aron, L., and Fielding, C. J. (1980) *Biochemistry* **19**, 3673–3677
35. Morton, R. E., and Zilversmit, D. B. (1981) *J. Biol. Chem.* **256**, 11992–11995
36. Newnham, H. H., and Barter, P. J. (1990) *Biochim. Biophys. Acta* **1044**, 57–64
37. Simonet, W. S., Bucay, N., Lauer, S. J., Wirak, D. O., Stevens, M. E., Weisgraber, K. H., Pitas, R. E., and Taylor, J. M. (1990) *J. Biol. Chem.* **265**, 10809–10812
38. Smith, J. D., Plump, A. S., Hayek, T., Walsh, A., and Breslow, J. L. (1990) *J. Biol. Chem.* **265**, 14709–14712

Brief Review

Cholesteryl Ester Transfer Protein
A Novel Target for Raising HDL and Inhibiting Atherosclerosis

Philip J. Barter, H. Bryan Brewer Jr, M. John Chapman, Charles H. Hennekens, Daniel J. Rader, Alan R. Tall

Abstract—Cholesteryl ester transfer protein (CETP) promotes the transfer of cholesteryl esters from antiatherogenic HDLs to proatherogenic apolipoprotein B (apoB)–containing lipoproteins, including VLDLs, VLDL remnants, IDLs, and LDLs. A deficiency of CETP is associated with increased HDL levels and decreased LDL levels, a profile that is typically antiatherogenic. Studies in rabbits, a species with naturally high levels of CETP, support the therapeutic potential of CETP inhibition as an approach to retarding atherogenesis. Studies in mice, a species that lacks CETP activity, have provided mixed results. Human subjects with heterozygous CETP deficiency and an HDL cholesterol level >60 mg/dL have a reduced risk of coronary heart disease. Evidence that atherosclerosis may be increased in CETP-deficient subjects whose HDL levels are not increased is difficult to interpret and may reflect confounding or bias. Small-molecule inhibitors of CETP have now been tested in human subjects and shown to increase the concentration of HDL cholesterol while decreasing that of LDL cholesterol and apoB. Thus, it seems important and timely to test the hypothesis in randomized trials of humans that pharmacological inhibition of CETP retards the development of atherosclerosis. (***Arterioscler Thromb Vasc Biol.* 2003;23:160-167.**)

Key Words: HDL ■ LDL ■ reverse cholesterol transport ■ genetic CETP deficiency

The possibility that cholesteryl ester transfer protein (CETP) might be proatherogenic and that inhibition of its activity might be antiatherogenic was first raised >10 years ago.[1] The potential atherogenicity of CETP relates to its ability to transfer cholesteryl esters from the antiatherogenic HDLs to the proatherogenic VLDL and LDL fractions. However, there is also evidence that CETP may be involved in reverse cholesterol transport (RCT). Thus, theoretically, CETP may be either proatherogenic or antiatherogenic. Most experimental evidence in animals favors a proatherogenic role for CETP and supports a view that inhibition of CETP is antiatherogenic.

It has also been suggested that CETP has the potential to inhibit atherogenesis by enhancing the rate of RCT, the pathway by which cholesterol in peripheral tissues is transported to the liver for elimination in the bile. This pathway involves an initial uptake of cell cholesterol by HDL, where it is esterified by lecithin:cholesterol acyltransferase (LCAT). A proportion of the HDL cholesteryl esters is delivered directly to the liver, whereas another proportion is transferred by CETP to LDL and VLDL. The cholesteryl esters in the VLDL/LDL pool are subsequently delivered to the liver via the LDL receptor pathway.

The available clinical data in humans are incomplete and do not permit a definite conclusion about the relation of CETP deficiency to the risk of coronary heart disease (CHD). In this article, we review the totality of evidence on CETP and suggest further research.

What Is CETP and What Does It Do?

CETP is a hydrophobic glycoprotein that is secreted mainly from the liver and that circulates in plasma, bound mainly to HDL.[2] It promotes the redistribution of cholesteryl esters, triglycerides, and, to a lesser extent, phospholipids between plasma lipoproteins. CETP transfers lipids from 1 lipoprotein particle to another in a process that results in equilibration of lipids between lipoprotein fractions.[3] Most of the cholesteryl esters in plasma originate in HDL in the reaction catalyzed by LCAT, and the majority of the triglycerides enter the plasma as a component of chylomicrons and VLDLs (triglyceride-rich lipoproteins [TRLs]). The overall effect of CETP is a net mass transfer of cholesteryl esters from HDLs to TRLs and LDLs and of triglycerides from TRLs to LDLs and HDLs (Figure 1). Thus, CETP-mediated transfers from HDL to VLDL and LDL provide a potential indirect pathway by

Received October 10, 2002; revision accepted December 13, 2002.

From the Hanson Institute and the Department of Cardiology (P.J.B.), Royal Adelaide Hospital, Adelaide, Australia; the National Heart, Lung, and Blood Institute (H.B.B. Jr), National Institutes of Health, Bethesda, Md; the National Institute for Health and Medical Research and Hopital de la Pitie (M.J.C.), Paris, France; the Preventive Cardiology and Lipid Clinic (D.J.R.), University of Pennsylvania Medical Center, Philadelphia; the Mount Sinai Medical Center–Miami Heart Institute and the Departments of Medicine and Epidemiology and of Public Health (C.H.H.), University of Miami School of Medicine, Miami, Fla; and the Division of Molecular Medicine (A.R.T.), Department of Medicine, Columbia University, New York, NY.

All authors contributed equally to this work and are listed in alphabetical order.

Correspondence to Prof Philip J. Barter, The Heart Research Institute, 145 Missenden Rd, Camperdown, Sydney, Australia 2050. E-mail p.barter@hri.org.au

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000054658.91146.64

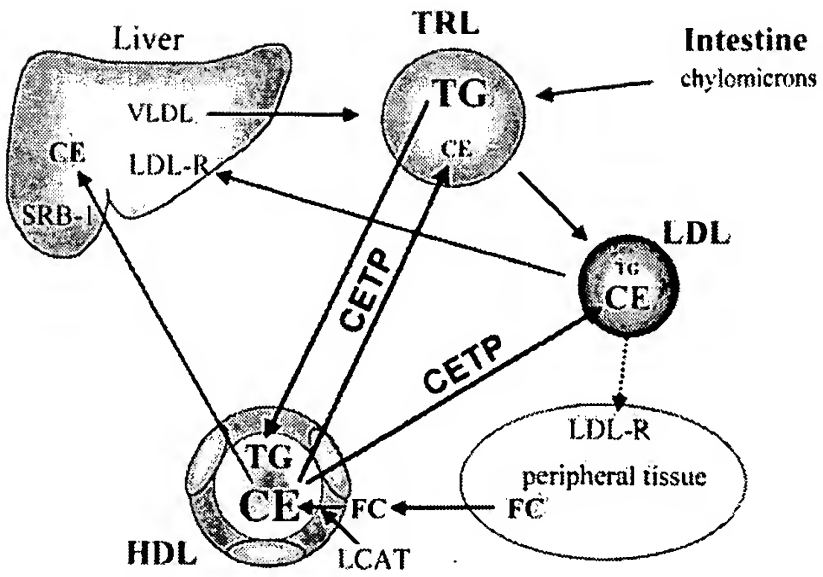

Figure 1. Role of CETP in plasma lipid transport. CETP promotes bidirectional transfers of cholesteryl esters (CE) and triglyceride (TG) between plasma lipoproteins. Because most of the CEs in plasma originate in HDL in the reaction catalyzed by LCAT and the majority of the TG enters plasma as a component of TRLs secreted either from the liver (VLDL) or intestine (chylomicrons), the overall effect of CETP is to promote a net mass transfer of CE from HDL to TRL and LDL and of TG from TRL to LDL and HDL. Pathways of RCT that deliver plasma CE to the liver include the hepatic uptake from HDL via the scavenger receptor B-1 (SRB-1) or the hepatic uptake of LDL via the LDL receptor (LDL-R).

which HDL cholesteryl esters can be delivered to the liver. In a high-CETP species such as the rabbit, this indirect pathway may account for as much as 70% of the cholesteryl esters that originate in HDL.[4]

Under usual conditions, the rate of CETP-mediated cholesteryl ester transfer is rapid relative to the rate of HDL and LDL catabolism.[3] As a consequence, the pools of cholesteryl esters in HDLs and LDLs approach equilibrium in vivo. Thus, whereas an increase in the activity of CETP beyond physiological levels would further increase the rate of bidirectional transfers between HDLs and LDLs, the effect on the distribution of cholesteryl esters between the 2 lipoprotein fractions would be relatively small. In contrast, if CETP were inhibited, then there would be a point beyond which its activity was rate limiting. Under these circumstances, the level of CETP activity may have an important role in determining the distribution of cholesteryl esters between LDLs and HDLs. In the case of transfers between HDLs and the much more rapidly catabolized VLDLs, the amount of CETP in plasma is almost certainly rate limiting under most conditions. Indeed, when the concentration of VLDLs is increased, the quantity of CETP is demonstrably the limiting factor in the rate at which cholesteryl esters are transferred from HDLs.[5]

When the level of VLDLs is normal, CETP-mediated transfers of HDL cholesteryl esters are directed preferentially to LDLs[6] (Figure 2). In contrast, when the concentration of VLDLs is increased, as in patients with type 2 diabetes, HDL cholesteryl esters are preferentially transferred by CETP to larger VLDL particles that become cholesterol rich and thus, potentially more atherogenic.[6] Transfers of cholesteryl esters to TRLs are enhanced in the postprandial state.[7,8] The rate of cholesteryl ester transfer to TRLs and LDLs and the mass of CETP are increased in patients with a range of atherogenic dyslipidemias.[9]

CETP contributes to an atherogenic lipid phenotype in several ways. It increases the cholesteryl ester content and thus, the atherogenicity of VLDLs. It also interacts with triglyceride lipases to generate small, dense LDLs[10] and HDLs[11] (Figure 2). The CETP-mediated reduction in HDL particle size is accompanied by the dissociation of lipid-poor apolipoprotein A-I (apoA-I) from the particle.[12-14] When the number of circulating acceptor VLDL and LDL particles is reduced in vivo by treatment with a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, there is retention of cholesteryl esters in the HDL fraction and an increase in the proportion of larger HDL_2 particles.[15]

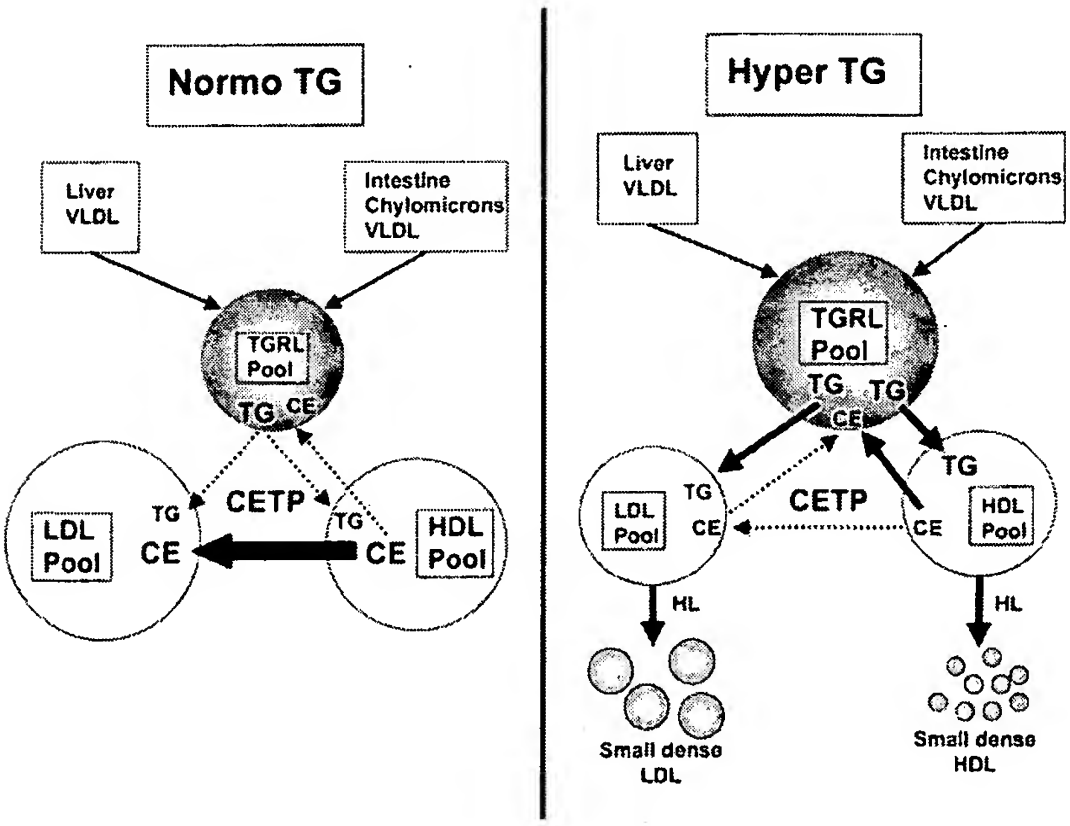

Figure 2. Effects of CETP in normotriglyceridemia (Normo TG) and hypertriglyceridemia (Hyper TG). The magnitude of the net flux of cholesteryl esters (CE) and TGs between lipoproteins is dependent, in large part, on the relative sizes of the TRL, LDL, and HDL pools. In Normo TG, net CE flux to LDLs from HDLs predominates, with minor transfer to TRLs. In contrast, in Hyper TG, increased particle numbers of large VLDLs exhibit elevated acceptor activity for CETP. Under these conditions, there are high net transfer rates of CEs from HDLs to TRLs and of TGs from TRLs to both HDLs and LDLs. TG-enriched LDLs and HDLs are substrates for hepatic lipase (HL) that hydrolyzes phospholipid (PL) and TGs to form small, dense LDL and small, dense HDL, respectively.

Natural Inhibitors of CETP in Human Plasma

The presence of natural inhibitors of CETP has been reported in human plasma. One such protein, initially referred to as lipid transfer inhibitor protein, has recently been identified as apoF.[16] This protein preferentially suppresses transfers involving LDLs but has less effect on transfers involving HDLs. CETP inhibitory activity within the HDL fraction has been identified as apoC-I.[17]

Role of CETP in the Development of Atherosclerosis in Animals

Expression of CETP in Transgenic Mice

Several species, including mice and rats, are naturally deficient in CETP. Introduction of the human *CETP* gene into mice results in a dose-related reduction in HDL levels and a small increase in VLDL and LDL cholesterol and apoB levels.[18,19] Mice are relatively resistant to the development of diet-induced atherosclerosis and must clearly accomplish RCT by pathways that do not involve CETP activity. In fact, in studies of bile salt and cholesterol-fed, C57-B16 mice, the introduction and expression of the simian *cetp* gene resulted in enhanced formation of fatty streak lesions compared with nonexpressing controls.[20] It was concluded that the enhancement of lesion development by CETP was secondary to a redistribution of cholesterol from HDLs to the VLDL/LDL fraction.

In another study,[21] the human *CETP* gene was introduced into chow-fed, apoE-knockout mice and also into Western diet–fed, LDL receptor–knockout mice, both of which develop spontaneous atherosclerosis. CETP expression in these animal models redistributed cholesterol from HDLs to the VLDL/LDL pool and also increased the development of atherosclerosis. These results again support a view that CETP is proatherogenic, possibly by virtue of reducing the concentration of HDLs and redistributing cholesteryl esters from HDLs to the VLDL/LDL pool. However, when the *CETP* gene was overexpressed in apoE-knockout mice that also overexpressed human apoA-I, the effect of CETP on the development of atherosclerosis was nonsignificant, despite being associated with a major reduction in the concentration of HDL cholesterol.[21]

There are some circumstances in which the expression of CETP in mice has been reported to be antiatherogenic rather than proatherogenic. An antiatherogenic effect of CETP has been observed in mice that have been engineered to overexpress human apoC-III.[22] These animals have high levels of triglyceride-rich remnant lipoproteins and develop small fatty-streak lesions. Introduction and expression of the *CETP* gene into these animals appeared to reduce the extent of the lesions.[22] In this and a more recent study in a hypertriglyceridemic mouse model (produced by streptozotocin-induced diabetes and lipoprotein lipase deficiency), CETP was antiatherogenic, probably by decreasing the concentration of cholesterol in small VLDL remnants.[23] Whether a comparable situation ever prevails in humans is uncertain. Another example of an antiatherogenic effect of CETP is seen in transgenic mice expressing human *LCAT*.[24] These *LCAT*-transgenic mice have an increased concentration of HDL cholesterol but paradoxically, also an increased susceptibility to atherosclerosis. Expression of simian *cetp* in these animals reduces the atherosclerosis.[25] It has been speculated, though not tested, that the cholesteryl ester–enriched HDLs that circulate in *LCAT*-transgenic mice may be less able to accept cholesterol from cells. If this were proved to be so, then the introduction of CETP would provide a means for transferring cholesteryl esters from the HDLs, possibly restoring their efficiency as acceptors of cell cholesterol and thus, as inhibitors of the development of atherosclerosis. This speculation is consistent with studies of rabbits, a species with a naturally high level of CETP. Transgenic expression of the human *LCAT* gene in rabbits increases the level of HDL cholesterol, reduces the concentration of LDL cholesterol, and, in contrast to mice, reduces diet-induced atherosclerosis.[26]

Inhibition of CETP in Rabbits

Rabbits are highly susceptible to the development of diet-induced atherosclerosis. Rabbits also have a naturally high level of CETP. Furthermore, it has been demonstrated in several rabbit models of atherosclerosis that inhibiting CETP results in a marked reduction in atherosclerosis.

In cholesterol-fed rabbits, the inhibition of CETP by injection of antisense oligodeoxynucleotides (ODNs) against CETP resulted in a reduction in CETP mRNA and mass in the liver, a reduction in plasma total cholesterol, and an increased concentration of HDL cholesterol.[27] There was also an increase in LDL receptor mRNA associated with the antisense ODNs. These changes were accompanied by a marked reduction in aortic cholesterol content as a marker of the extent of atherosclerosis.

It is also possible to inhibit CETP in vivo by infusing anti-CETP antibodies into rabbits.[28] This study showed an effect of CETP on the distribution of cholesteryl esters between HDLs and VLDLs/LDLs but did not investigate the effects of inhibition on the development of atherosclerosis. In a more recent report,[29] a vaccine approach has been used to generate auto-antibodies against CETP in vivo in rabbits. In a study of cholesterol-fed rabbits, animals that were immunized against CETP had a reduced plasma activity of CETP and a substantial increase in the concentration of HDL cholesterol. They also had a modest decrease in LDL cholesterol concentration and a significant reduction in aortic atherosclerotic lesions. This study demonstrates that long-term inhibition of CETP is not only possible but also, at least in rabbits, reduces the susceptibility to atherosclerosis.

A newly developed chemical inhibitor of CETP has been used in another recent study of cholesterol-fed rabbits.[30] This inhibitor reduced CETP activity in rabbits by >90%, almost doubled the level of HDL cholesterol, and decreased the non-HDL cholesterol by ≈50%. There was an accompanying 70% reduction in atherosclerotic lesions in the aortas of these animals. It was not possible to determine the relative importance of the increased HDL versus the decreased LDL in the reduction of atherosclerosis observed in these rabbit studies. It was speculated that short-term treatment of humans with the same CETP inhibitor would result in a 40% to 45% increase in HDL cholesterol and a 15% to 20% decrease in LDL cholesterol.

CETP, Lipoprotein Metabolism, and Atherosclerosis in Humans

Genetic CETP Deficiency in Humans and Effects on Lipoprotein Metabolism

Several mutations of the *CETP* gene have been identified as a cause of CETP deficiency and elevated levels of HDL cholesterol. These include a G-to-A mutation at the +1 position of intron 14,[1,31] a mutation that is present in up to 2% of the overall Japanese population[32,33] and in as many as 27% of people in the Omagari area of Japan.[34] Individuals homozygous for this mutations have no measurable CETP mass or activity in their plasma but do have elevated concentrations of both HDL lipids (cholesterol and phospholipid; 3- to 4-fold) and apoA-I (1.7-fold).[31] A second common functional mutation of the *CETP* gene, present in up to 7% of the Japanese population, involves a D-to-G substitution at the 442 position of exon 15[33] that also results in increased HDL levels. However, subjects homozygous for this D442G mutation have only a partial rather than a complete CETP deficiency, and HDL levels are elevated 1- to 2- fold .[33]

Subjects with a homozygous CETP deficiency have elevated concentrations of HDL cholesterol, apoA-I, apoA-II, and apoE. The HDL fraction is especially enriched in larger, less dense, cholesteryl ester and apoE-enriched HDL_2 particles.[35] The increased HDL concentration is due to a reduction in the rate of catabolism, with a markedly delayed catabolism of both apoA-I and apoA-II.[36] In contrast, the synthesis of both apoA-I and apoA-II is similar to that in control subjects.[36] Despite the delayed catabolism of apoA-I and apoA-II, it has not been established whether the net flux of cholesterol to the liver is decreased in patients with CETP deficiency. In fact, there are no data to suggest that the rate of HDL apolipoprotein turnover is directly correlated with the net flux of cholesterol to the liver.

The ability of HDLs from homozygous CETP-deficient individuals to promote the efflux of cholesterol from macrophages has been investigated.[37] The large, apoE-rich HDLs were poor acceptors of macrophage cholesterol, but the apoE-free HDL_2 and the HDL_3 from these subjects functioned normally as acceptors of macrophage cholesterol.

In addition to the elevation of HDL that accompanies CETP deficiency, in homozygotes there is a substantial reduction (≈40%) in the concentration of LDL cholesterol and apoB.[31] These individuals have a polydisperse LDL fraction extending across the whole LDL density range.[38,39] Human CETP deficiency is associated with both a decreased rate of production of apoB and an increase in its rate of catabolism, consistent with an upregulation of the LDL receptor in these subjects.[40] When preparations of LDL isolated from CETP-deficient patients and from control subjects were injected into control subjects, the CETP-deficient LDL had a delayed catabolism.[40] This result is consistent with the observation of decreased affinity of the CETP-deficient LDLs for the LDL receptor.[41] One plausible explanation consistent with both the LDL kinetic studies and the LDL receptor–binding studies is that the LDLs from CETP deficient patients are altered. However, these results are difficult to interpret in terms of atherogenic potential, because any rapidly cleared particles would be underrepresented. Furthermore, it is not known whether the net mass of cholesteryl esters cleared is altered when the particle number is reduced.

Atherosclerosis in CETP-Deficient Human Subjects

The relation between mutations of the *CETP* gene and susceptibility to premature atherosclerosis is complex. In the Honolulu Heart Study, many of the participants of Japanese origin were heterozygous for the D442G mutation in the *CETP* gene and had reduced levels of CETP.[42] Those in whom the CETP deficiency coincided with HDL cholesterol concentrations of 41 to 60 mg/dL (1.0 to 1.5 mmol/L) had an apparent 50% increase in CHD, although the total number of events in this group was too low to enable firm conclusions to be drawn. When the CETP deficiency was associated with higher HDL cholesterol levels (>60 mg/dL; >1.5 mmol/L), there was no evidence of an increase in CHD. Rather, in this subgroup, there was a low rate of CHD, comparable to that observed in the subjects in whom an elevated HDL cholesterol level was not associated with a deficiency of CETP.

A recent analysis of the 7-year prospective data from the Honolulu Heart Study revealed no statistically significant relation between heterozygous mutations of *CETP* and CHD or stroke, a finding at variance with the previous suggestion from prevalence data of increased risks (A.R Tall et al, in preparation). In fact, prospective analysis of the data has revealed a nonsignificant trend toward a lower incidence of cardiovascular events in subjects with CETP mutations.

Results similar to those from the Honolulu Heart Study were found in another study of Japanese subjects.[43] Individuals with HDL cholesterol levels >80 mg/dL were genotyped for intron 14 and D442G mutations. Subjects with and without *CETP* gene mutations (homozygous or heterozygous) who had HDL cholesterol levels >80 mg/dL had a very low risk of CHD. Given that the plasma level of CETP is, if anything, lower in deficient subjects with the highest HDL cholesterol levels,[42] it is difficult to implicate CETP deficiency per se as a cause of increased CHD. It is possible that a deficiency of CETP is protective, so long as it induces a substantial increase in HDL cholesterol.

HDLs may inhibit the development of atherosclerosis by mechanisms independent of their involvement in RCT. These include antioxidant[44] and anti-inflammatory[45,46] properties of HDL, both of which have an antiatherogenic potential that may be enhanced when the concentration of HDL is increased in CETP-deficient states.

CETP Polymorphisms and Susceptibility to Atherosclerosis in Humans

There are several reported restriction fragment length polymorphisms (RFLPs) in the human *CETP* gene (Table 1). Although many of these studies have demonstrated associations between *CETP* single-nucleotide polymorphisms and small changes in plasma CETP and HDL levels, the relation between these polymorphisms and susceptibility to atherosclerosis is variable (Table 2). Indeed, no consistent picture has emerged from numerous studies examining the relation

TABLE 1. Mutations or Polymorphisms of the Human CETP Gene

Variation	Location
C-631A	Promoter
C-629A	Promoter
TaqIB	Intron 1
A38X	**Exon 2**
G181X	**Exon 6**
+8 C→T	Intron 7
Msp I	Intron 8
R268X	Exon 9
Q309X	Exon 10
Int10G	Intron 10
A373P	Exon 12
I405V	Exon 12
Int14A	**Intron 14**
D442G	**Exon 15**
R451Q	Exon 15
+524 G→T	3' Region

Mutations or polymorphisms in bold are those that directly affect CETP function or expression. Sources: Bruce et al[59] and Corbex et al.[57]

between polymorphisms and atherosclerotic cardiovascular disease.

The best-studied RFLP is TaqIB in intron 1. In 1 study, the TaqIB polymorphism accounted for 5.8% of the variance in HDL cholesterol.[47] Subjects homozygous for the *B1* allele in the Framingham Offspring Study[48] had higher levels of CETP and lower levels of HDL cholesterol when compared with either *B1B2* or *B2B2* subjects. Men with the *B2* allele appeared to have a reduced risk of CHD, although there was no significant association in women. A similar result was obtained in the VA-HIT study in men with CHD and low HDL.[49] The *TaqIB* genotype is associated with some of the variation in response to statin therapy, as measured by the progression of coronary atherosclerosis by angiography.[50] Several other studies investigating the effects of the TaqIB polymorphism are summarized in Table 1. Overall, studies of the TaqIB polymorphism have been consistent and supportive of a view that a lower level of CETP is associated with increased HDL cholesterol and possibly a decreased risk of CHD in men.

Results from studies of other polymorphisms have not demonstrated consistent associations between *CETP* genotype and either atherosclerosis or CHD. In an investigation of the common I405V polymorphism among 576 men of Japanese ancestry, plasma CETP concentrations were 1.95 μg/mL for those with the *II* genotype, 1.91 μg/mL for the *IV* genotype, and 1.77 μg/mL for the *VV* genotype. HDL levels were highest among those with the *VV* genotype and lowest among those with the *II* genotype,[51] although the increase was significant only in *VV* homozygotes with plasma triglyceride levels >165 mg/dL (1.9 mmol/L). There were no differences in CHD risk among the 3 genotypes in the total population, although in a subset of individuals with high plasma triglyceride levels, the CHD risk was higher in those with the *VV* genotype than in those with the *IV* or *II* genotype (38% vs 27% vs 18%, respectively; $P<0.05$ for an interaction of genotype and plasma triglyceride levels). Though not significant, the apparent trend was opposite in those with triglyceride levels <165 mg/dL (1.9 mmol/L).

In an analysis of the I405V polymorphism in participants of the Copenhagen City Heart Study, women heterozygous or homozygous for the presence of valine at position 405 had increased levels of HDL cholesterol but also a paradoxical increased risk of CHD.[52] There was no association between the I405V polymorphism and either HDL cholesterol levels or CHD risk in the men in this study. In another investigation of the I405V polymorphism in the Stanislas cohort study, the I405V polymorphism was not related to any lipid parameter.[53]

A further report from Copenhagen focused on the A373P and R451Q polymorphisms.[54] All carriers of 451Q also carried the 373P allele. Carriers of the 451Q/373P alleles had reduced levels of HDL cholesterol and a paradoxically lower CHD risk when compared with the overall study population. The reasons for these apparently conflicting observations remain unclear but may be related, at least in part, to uncontrolled confounding by other genes or environmental factors.

Another possibly functional polymorphism has recently been reported in the promoter region of the *CETP* gene (−629A/C).[55] The mass of CETP was higher and the concentration of HDL cholesterol lower in individuals with the *A* allele than in those with the *C* allele, although the HDL cholesterol concentration was not correlated significantly with CETP mass. In studies of possible mechanisms whereby the polymorphism influenced CETP mass, it was found that repressor transcription factors that bound to the *A* allele did not bind to the *C* allele. An interaction between CETP polymorphisms and lifestyle factors is suggested by 2 recent reports that alcohol consumption greatly influences the apparent effects of several of the CETP polymorphisms.[56,57]

Inhibition of CETP in Humans: Effects on Plasma Lipoproteins

A newly developed chemical inhibitor of CETP, JTT-705, has been tested in humans.[58] In a 4-week, phase II, dose-response study, 198 healthy individuals with mild hyperlipidemia were randomly assigned to 300, 600, or 900 mg/d of JTT-705 or to placebo.[58] Treatment with the highest dose was associated with a highly significant 37% decrease in CETP activity, a 34% increase in HDLs, and a 7% decrease in LDLs. Levels of triglycerides, phospholipid transfer protein, and LCAT were unchanged. Doses up to 900 mg/d were safe and well tolerated but were significantly more likely than placebo to cause mild gastrointestinal side effects.

Conclusions

Expression of CETP in transgenic mice, a species that is naturally deficient in this protein, has yielded inconsistent results, with reports of both an increased susceptibility and protection against atherosclerosis in different models. In contrast, the effects in rabbits have been remarkably consistent and strongly support the therapeutic potential of CETP

TABLE 2. Relationship of Human CETP Polymorphisms to Atherosclerosis and CHD

Study	No. of Subjects	CETP Measure	Effect on Atherosclerosis
Honolulu Heart Program (1996),[42] cross-sectional	3469 men	CETP gene polymorphism, primarily D442G	50% increased risk of CHD among men with CETP mutation and HDL 41 to 60 mg/dL; similar risks among those with and without mutation at high HDL (>60 mg/dL)
Omagari population (1997),[34] cross-sectional	879 men and women	No direct measure of CETP mutations	U-shaped curve of ischemic changes on ECG–highest among men without mutation and low HDL and among men with mutation and high HDL; however, this study was not controlled for alcohol intake
Kochi prefecture (1998),[43] cross-sectional	48 531 men and women	Common CETP polymorphisms	Prevalence of CHD lower among those with HDL >60 mg/dL; no difference in prevalence among those with high HDL and mutation or high HDL and no mutation
Honolulu Heart Program (1998)[51] Cross-sectional	576 men	I405V polymorphism	HDL levels highest among men with VV genotype; possible increase in prevalence of CHD among VV men with TGs>165 mg/dL.
Kakko et al[56] (2000), cross-sectional	515 men and women	I405V polymorphism	Increased intima media thickness of carotid arteries among men with VV genotype who were heavy drinkers; no increased risk among other groups of men or among women
		R451Q polymorphism	Decreased intima media thickness of carotid arteries among men
Copenhagen City Heart Study[52] (2000), cross-sectional and case control	Men and women: 9168 healthy and 946 with IHD	I405V polymorphism	Increased risk of CHD among women with the IV or VV genotype not using HRT; no association of genotype and CHD risk among men
Framingham Offspring Study[48] (2000), cross-sectional	2916 men and women	B2 allele of TaqIB polymorphism	Possible decreased risk of CHD among men with the B2 allele, although this may have been due to association between B2 allele and HDL levels; no effect in women
Corbex et al[57] (2000), case-control	568 men and women with MI and 668 controls	10 polymorphisms: −631, −629, intron1, intron7, C373, C405, C451, intron12, intron16, and +524	Variable influences on HDL; Reduced risk of MI among subjects who drank >75g/day and who were homozygous for the −629A allele
Copenhagen City Heart Study[54] (2000), cross-sectional	8467 men and women	R451Q and A373P polymorphisms	Minor alleles associated with 10% lowering of HDL, no significant effect on IHD in men, effect in women only achieves significance when adjusted for HDL levels and segregated by menopausal/HRT status
Zhuang et al.[60] (2001), case-control	227 MI and stroke and 335 controls	Concentration of CETP	Higher levels of CETP among cases than controls
Stanislas cohort[61] (2001), cross-sectional	161 healthy men and women	I405V polymorphism	Trend toward increased intima-media thickness among men homozygous for the Ile405 allele
Meguro et al[62] (2001), case study	182 type 2 DM men and women	TaqIB polymorphism	Increased CETP and macroangiopathy among those with the B1/B1 genotype
Goto et al[63] (2001), cross-sectional	110 consecutive angiography patients	TaqIB polymorphism	No overall correlation between CETP mass and HDL levels or coronary atherosclerosis; lower CETP mass among individuals with B2B2 genotype in the lowest coronary score quartile
Reykjavik Study[64] (2001), case-control	388 male MI survivors and 794 healthy controls	TaqIB and −629A/C polymorphisms	B1B1 homozygotes had lower HDL and higher MI risk (OR=1.44, 95% CI: 1.10–1.87, $P<0.01$) compared with other genotypes combined, with MI experienced 2 years earlier in life
Physicians' Health Study[65] (2002)	384 men with first MI	TaqIB polymorphism	After adjustment for coronary risk factors (but not HDL), RR for first MI not significantly different across the three genotypes; men with high HDL (>53 mg/dL) were at lower risk of first MI regardless of CETP genotype

DM indicates diabetes mellitus; ECG, electrocardiogram; HRT, hormone replacement therapy; IHD, ischemic heart disease; MI, myocardial infarction; RR, relative risk.

inhibition for blunting or even halting the development and progression of atherosclerosis.

The available human data, though sparse, generally support the hypothesis that CETP deficiency, especially when associated with a high HDL level, is antiatherogenic. Those studies suggesting that atherosclerosis is increased in some individuals with a heterozygous CETP deficiency may reflect chance owing to small sample sizes, confounding by other risk factors, or biases inherent in the study design. There are no reliable data indicating that CETP inhibition in humans

would be unsafe. Indeed, CETP inhibitors may provide a powerful therapeutic approach to raising HDL levels, lowering LDL levels, and reducing the development of atherosclerosis in humans. However, such a proposition is based on circumstantial evidence and will remain hypothetical until subjected to direct testing. The recent negative trials with postmenopausal estrogens (that also increase HDL and lower LDL levels) highlight the pitfalls of drawing therapeutic conclusions from circumstantial evidence.

Thus, the hypothesis that pharmacological inhibition of CETP reduces the risk of atherosclerosis should be directly tested in randomized trials with either surrogate or clinical end points. Such trials should be of sufficient size, dose, and duration to reliably test the hypothesis. Further multidisciplinary research efforts into the metabolism of HDL, the precise roles played by CETP, and its interactions with other components of lipid transport and metabolism are also necessary to complete the totality of evidence.

References

1. Brown ML, Inazu A, Hesler CB, Agellon LB, Mann C, Whitlock ME, Marcel YL, Milne RW, Koizumi J, Mabuchi H, Tall AR. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature*. 1989;342:448–451.
2. Tall AR. Plasma cholesteryl ester transfer protein. *J Lipid Res*. 1993;34:1255–1274.
3. Barter PJ, Hopkins CJ, Calvert GD. Transfers and exchanges of esterified cholesterol between plasma lipoproteins. *Biochem J*. 1982;208:1–7.
4. Goldberg DI, Beltz WF, Pittman RC. Evaluation of pathways for the cellular uptake of high density lipoprotein cholesteryl esters in rabbits. *J Clin Invest*. 1991;87:331–346.
5. Mann CJ, Yen FT, Grant AM, Bihain BE. Mechanism of cholesteryl ester transfer in hypertriglyceridemia. *J Clin Invest*. 1991;88:2059–2066.
6. Guerin M, Le Goff W, Lassel TS, Van Tol A, Steiner G, Chapman MJ. Atherogenic role of elevated CE transfer from HDL to VLDL (1) and dense LDL in type 2 diabetes: impact of the degree of hypertriglyceridemia. *Arterioscler Thromb Vasc Biol*. 2001;21:282–288.
7. Contacos C, Barter PJ, Vrga L, Sullivan DR. Cholesteryl ester transfer in hypercholesterolaemia: fasting and postprandial studies with and without pravastatin. *Atherosclerosis*. 1998;141:87–98.
8. Guerin M, Egger P, Soudant C, Le Goff W, van Tol A, Dupuis R, Chapman MJ. Cholesteryl ester flux from HDL to VLDL-1 is preferentially enhanced in type IIB hyperlipidemia in the postprandial state. *J Lipid Res*. 2002;43:1652–1660.
9. McPherson R, Mann CJ, Tall AR, Hogue M, Martin L, Milne RW, Marcel YL. Plasma concentration of cholesteryl ester transfer protein in hyperlipoproteinemia: relation to cholesteryl ester transfer protein activity and other variables. *Arterioscler Thromb*. 1991;11:797–804.
10. Chung BH, Segrest JP, Franklin F. In vitro production of β-very low density lipoproteins and small, dense low density lipoproteins in mildly hypertriglyceridemic plasma: role of activities of lecithin:cholesterol acyltransferase, cholesteryl ester transfer protein and lipoprotein lipase. *Atherosclerosis*. 1998;141:209–225.
11. Newnham HH, Barter PJ. Synergistic effects of lipid transfers and hepatic lipase in the formation of very small high density lipoproteins during incubation of human plasma. *Biochim Biophys Acta*. 1990;1044:57–64.
12. Rye K-A, Hime NJ, Barter PJ. Evidence that CETP-mediated reductions in reconstituted high density lipoprotein size involve particle fusion. *J Biol Chem*. 1997;272:5953–5960.
13. Rye K-A, Clay MA, Barter PJ. Remodelling of high density lipoproteins by plasma factors. *Atherosclerosis*. 1999;145:227–238.
14. Liang H-Q, Rye K-A, Barter PJ. Dissociation of lipid-free apolipoprotein A-I from high density lipoproteins. *J Lipid Res*. 1994;35:1187–1199.
15. Guerin M, Lassel TS, Le Goff W, Farnier M, Chapman MJ. Action of atorvastatin in combined hyperlipidemia: preferential reduction of cholesteryl ester transfer from HDL to VLDL1 particles. *Arterioscler Thromb Vasc Biol*. 2000;20:189–197.
16. Wang X, Driscoll DM, Morton RE. Molecular cloning and expression of lipid transfer inhibitor protein reveals its identity with apolipoprotein F. *J Biol Chem*. 1999;274:1814–1820.
17. Gautier T, Masson D, de Barros JP, Athias A, Gambert P, Aunis D, Metz-Boutigue MH, Lagrost L. Human apolipoprotein C-I accounts for the ability of plasma high density lipoproteins to inhibit the cholesteryl ester transfer protein activity. *J Biol Chem*. 2000;275:37504–37509.
18. Agellon LB, Walsh A, Hayek T, Moulin P, Jiang XC, Shelanski SA, Breslow JL, Tall AR. Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. *J Biol Chem*. 1991;266:10796–10801.
19. Jiang XC, Masucci-Magoulas L, Mar J, Lin M, Walsh A, Breslow JL, Tall A. Down-regulation of mRNA for the low density lipoprotein receptor in transgenic mice containing the gene for human cholesteryl ester transfer protein: mechanism to explain accumulation of lipoprotein B particles. *J Biol Chem*. 1993;268:27406–27412.
20. Marotti KR, Castle CK, Boyle TP, Lin AH, Murray RW, Melchior GW. Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature*. 1993;364:73–75.
21. Plump AS, Masucci-Magoulas L, Bruce C, Bisgaier CL, Breslow JL, Tall AR. Increased atherosclerosis in apoE and LDL receptor gene knock-out mice as a result of human cholesteryl ester transfer protein transgene expression. *Arterioscler Thromb Vasc Biol*. 1999;19:1105–1110.
22. Hayek T, Masucci-Magoulas L, Jiang X, Walsh A, Rubin E, Breslow JL, Tall AR. Decreased early atherosclerotic lesions in hypertriglyceridemic mice expressing cholesteryl ester transfer protein transgene. *J Clin Invest*. 1995;96:2071–2074.
23. Kako Y, Masse M, Huang LS, Tall AR, Goldberg IJ. Lipoprotein lipase deficiency and CETP in streptozotocin-treated apoB-expressing mice. *J Lipid Res*. 2002;43:872–877.
24. Berard AM, Foger B, Remaley A, Shamburek R, Vaisman BL, Talley G, Paigen B, Hoyt RF Jr, Marcovina S, Brewer HB Jr, Santamarina-Fojo S. High plasma HDL concentrations associated with enhanced atherosclerosis in transgenic mice overexpressing lecithin-cholesteryl acyltransferase. *Nat Med*. 1997;3:744–749.
25. Foger B, Chase M, Amar MJ, Vaisman BL, Shamburek RD, Paigen B, Fruchart-Najib J, Paiz JA, Koch CA, Hoyt RF, Brewer HB Jr, Santamarina-Fojo S. Cholesteryl ester transfer protein corrects dysfunctional high density lipoproteins and reduces aortic atherosclerosis in lecithin cholesterol acyltransferase transgenic mice. *J Biol Chem*. 1999;274:36912–36920.
26. Hoeg JM, Santamarina-Fojo S, Berard AM, Cornhill JF, Herderick EE, Feldman SH, Haudenschild CC, Vaisman BL, Hoyt RF Jr, Demosky SJ Jr, Kauffman RD, Hazel CM, Marcovina SM, Brewer HB Jr. Overexpression of lecithin:cholesterol acyltransferase in transgenic rabbits prevents diet-induced atherosclerosis. *Proc Natl Acad Sci U S A*. 1996;93:11448–11453.
27. Sugano M, Makino N, Sawada S, Otsuka S, Watanabe M, Okamoto H, Kamada M, Mizushima A. Effect of antisense oligonucleotides against cholesteryl ester transfer protein on the development of atherosclerosis in cholesterol-fed rabbits. *J Biol Chem*. 1998;273:5033–5036.
28. Whitlock ME, Swenson TL, Ramakrishnan R, Leonard MT, Marcel YL, Milne RW, Tall AR. Monoclonal antibody inhibition of cholesteryl ester transfer protein activity in the rabbit. *J Clin Invest*. 1989;84:129–137.
29. Rittershaus CW, Miller DP, Thomas LJ, Picard MD, Honan CM, Emmett CD, Pettey CL, Adari H, Hammond RA, Beattie DT, Callow AD, Marsh HC, Ryan US. Vaccine-induced antibodies inhibit CETP activity in vivo and reduce aortic lesions in a rabbit model of atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2000;20:2106–2112.
30. Okamoto H, Yonemori F, Wakitani K, Minowa T, Maeda K, Shinkai H. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature*. 2000;406:203–207.
31. Inazu A, Brown ML, Hesler CB, Agellon LB, Koizumi J, Takata K, Maruhama Y, Mabuchi H, Tall AR. 1: Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. *N Engl J Med*. 1990;323:1234–1238.
32. Akita H, Chiba H, Tsuchihashi K, Tsuji M, Kumagai M, Matsuno K, Kobayashi K. Cholesteryl ester transfer protein gene: two common mutations and their effect on plasma high-density lipoprotein cholesterol content. *J Clin Endocrinol Metab*. 1994;79:1615–1618.
33. Inazu A, Jiang XC, Haraki T, Yagi K, Kamon N, Koizumi J, Mabuchi H, Takeda R, Takata K, Moriyama Y, Doi M, Tall A. Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J Clin Invest*. 1994;94:1872–1882.
34. Hirano K, Yamashita S, Nakajima N, Arai T, Maruyama T, Yoshida Y, Ishigami M, Sakai N, Kameda-Takemura K, Matsuzawa Y. Genetic cholesteryl ester transfer protein deficiency is extremely frequent in the

Omagari area of Japan. *Arterioscler Thromb Vasc Biol.* 1997;17: 1053–1059.
35. Yamashita S, Sprecher DL, Sakai N, Matsuzawa Y, Tarui S, Hui DY. Accumulation of apolipoprotein E-rich high density lipoproteins in hyperalphalipoproteinemic human subjects with deficiency of cholesteryl ester transfer protein. *J Clin Invest.* 1990;86:688–695.
36. Ikewaki K, Rader DJ, Sakamoto T, Nishiwaki M, Wakimoto N, Schaefer JR, Ishikawa T, Fairwell T, Zech LA, Nakamura H, Nagano M, Brewer HB. Delayed catabolism of high density lipoprotein apolipoprotein A-I and A-II in human cholesteryl ester transfer protein deficiency. *J Clin Invest.* 1993;92:1650–1658.
37. Ishigami M, Yamashita S, Sakai N, Arai T, Hirano K, Hiraoka H, Kameda-Takemura K, Matsuzawa Y. Large and cholesteryl ester-rich high-density lipoproteins in cholesteryl ester transfer protein (CETP) deficiency cannot protect macrophages from cholesterol accumulation induced by acetylated low-density lipoproteins. *J Biochem (Tokyo).* 1994; 116:257–262.
38. Yamashita S, Matsuzawa Y, Okazaki M, Kako H, Yasugi T, Akioka H, Hirano K, Tarui S. Small polydisperse low density lipoproteins in familial hyperalphalipoproteinemia with complete deficiency of cholesteryl ester transfer activity. *Atherosclerosis.* 1988;70:7–12.
39. Sakai N, Matsuzawa Y, Hirano K, Yamashita S, Nozaki S, Ueyama Y, Kubo M, Tarui S. Detection of two species of low density lipoprotein particles in cholesteryl ester transfer protein deficiency. *Arterioscler Thromb.* 1991;11:71–79.
40. Ikewaki K, Nishiwaki M, Sakamoto T, Ishikawa T, Fairwell T, Zech LA, Nagano M, Nakamura H, Brewer HB Jr, Rader DJ. Increased catabolic rate of low density lipoproteins in humans with cholesteryl ester transfer protein deficiency. *J Clin Invest.* 1995;96:1573–1581.
41. Sakai N, Yamashita S, Hirano K, Ishigami M, Arai T, Kobayashi K, Funahashi T, Matsuzawa Y. Decreased affinity of low density lipoprotein (LDL) particles for LDL receptors in patients with cholesteryl ester transfer protein deficiency. *Eur J Clin Invest.* 1995;25:332–339.
42. Zhong S, Sharp DS, Grove JS, Bruce C, Yano K, Curb JD, Tall AR. Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. *J Clin Invest.* 1996;97:2917–2923.
43. Moriyama Y, Okamura T, Inazu A, Doi M, Iso H, Mouri Y, Ishikawa Y, Suzuki H, Iida M, Koizumi J, Mabuchi H, Komachi Y. A low prevalence of coronary heart disease among subjects with increased high density lipoprotein cholesterol levels, including those with plasma cholesteryl ester transfer protein deficiency. *Prev Med.* 1998;27:659–667.
44. Mackness MI, Abbott C, Arrol S, Durrington PN. The role of high-density lipoprotein and lipid-soluble antioxidant vitamins in inhibiting low-density lipoprotein oxidation. *Biochem J.* 1993:294:829–834.
45. Barter PJ, Rye K-A. High density lipoproteins and coronary heart disease. *Atherosclerosis.* 1996;121:1–12.
46. Dimayuga P, Zhu J, Oguchi S, Chyu KY, Xu ZOH, Yano J, Shah PK, Nillson J, Cercek B. Reconstituted HDL containing human apolipoprotein A-I reduces VCAM-1 expression and neointima formation following periadventitial cuff-induced carotid injury in apoE null mice. *Biochem Biophys Res Commun.* 1999:264:465–468.
47. Corella D, Saiz C, Guillen M, Portoles O, Mulet F, Gonzalez JI, Ordovas JM. Association of TaqIB polymorphism in the cholesteryl ester transfer protein gene with plasma lipid levels in a healthy Spanish population. *Atherosclerosis.* 2000;152:367–376.
48. Ordovas JM, Cupples LA, Corella D, Otvos JD, Osgood D, Martinez A, Lahoz C, Coltell O, Wilson PW, Schaefer EJ. Association of cholesteryl ester transfer protein-*Taq*IB polymorphism with variations in lipoprotein subclasses and coronary heart disease risk. *Arterioscler Thromb Vasc Biol.* 2000;20:1323–1329.
49. Brousseau ME, O'Connor JJ Jr, Ordovas JM, Collins D, Otvos JD, Massov T, McNamara JR, Rubins HB, Robins SJ, Schaefer EJ. Cholesteryl ester transfer protein TaqI *B2B2* genotype is associated with higher HDL cholesterol levels and lower risk of coronary heart disease end points in men with HDL deficiency: Veterans Affairs HDL Cholesterol Intervention Trial. *Arterioscler Thromb Vasc Biol.* 2002;22: 1148–1154.
50. Kuivenhoven JA, Jukema JW, Zwinderman AH, de Knijff P, McPherson R, Bruschke AV, Lie KI, Kastelein JJ. The role of a common variant of the cholesteryl ester transfer protein gene in the progression of coronary atherosclerosis: the Regression Growth Evaluation Statin Study Group. *N Engl J Med.* 1998;338:86–93.
51. Bruce C, Sharp DS, Tall AR. Relationship of HDL and coronary heart disease to a common amino acid polymorphism in the cholesteryl ester transfer protein in men with and without hypertriglyceridemia. *J Lipid Res.* 1998;39:1071–1078.
52. Agerholm-Larsen B, Nordestgaard BG, Steffensen R, Jensen G, Tybjaerg-Hansen A. Elevated HDL cholesterol is a risk factor for ischemic heart disease in white women when caused by a common mutation in the cholesteryl ester transfer protein gene. *Circulation.* 2000;101: 1907–1912.
53. Pallaud C, Gueguen R, Sass C, Grow M, Cheng S, Siest G, Visvikis S. Genetic influences on lipid metabolism trait variability within the Stanislas Cohort. *J Lipid Res.* 2001;42:1879–1890.
54. Agerholm-Larsen B, Tybjaerg-Hansen A, Schnohr P, Steffensen R, Nordestgaard BG. Common cholesteryl ester transfer protein mutations, decreased HDL cholesterol, and possible decreased risk of ischemic heart disease: the Copenhagen City Heart Study. *Circulation.* 2000;102: 2197–2203.
55. Dachet C, Poirier O, Cambien F, Chapman J, Rouis M. New functional promoter polymorphism, CETP/-629, in cholesteryl ester transfer protein (CETO) gene related to CETP mass and high density lipoprotein levels: role of Sp1/Sp3 in transcriptional regulation. *Arterioscler Thromb Vasc Biol.* 2000;20:507–515.
56. Kakko S, Tamminen M, Paivansalo M, Kauma H, Rantala AO, Lilja M, Reunanen A, Kesaniemi YA, Savolainen MJ. Cholesteryl ester transfer protein gene polymorphisms are associated with carotid atherosclerosis in men. *Eur J Clin Invest.* 2000;30:18–25.
57. Corbex M, Poirier O, Fumeron F, Betoulle D, Evans A, Ruidavets JB, Arveiler D, Luc G, Tiret L, Cambien F. Extensive association analysis between the CETP gene and coronary heart disease phenotypes reveals several putative functional polymorphisms and gene-environment interaction. *Genet Epidemiol.* 2000;19:64–80.
58. de Grooth GJ, Kuivenhoven JA, Stalenhoef AF, de Graaf J, Zwinderman AH, Posma JL, van Tol A, Kastelein JJ. Efficacy and safety of a novel cholesteryl ester transfer protein inhibitor, JTT-705, in humans: a randomized phase II dose-response study. *Circulation.* 2002;105: 2159–2165.
59. Bruce C, Chouinard RA, Jr, Tall AR. Plasma lipid transfer proteins, high-density lipoproteins, and reverse cholesterol transport. *Annu Rev Nutr.* 1998;18:297–330.
60. Zhuang Y, Wang J, Qiang H, Li Y, Lui X, Li L, Chen G. Serum cholesteryl ester transfer protein concentrations in healthy Chinese subjects and cardio-cerebrovascular disease patients. *Clin Chim Acta.* 2001;305:19–25.
61. Pallaud C, Sass C, Zannad F, Siest G, Visvikis S. APOC3, CETP, fibrinogen, and MTHFR are genetic determinants of carotid intima-media thickness in healthy men (the Stanislas cohort). *Clin Genet.* 2001;59: 316–324.
62. Meguro S, Takei I, Murata M, Hirose H, Takei N, Mitsuyoshi Y, Ishii K, Oguchi S, Shinohara J, Takeshita E, Watanabe K, Saruta T. Cholesteryl ester transfer protein polymorphism associated with macroangiopathy in Japanese patients with type 2 diabetes. *Atherosclerosis.* 2001;156: 151–156.
63. Goto A, Sasai K, Suzuki S, Fukutomi T, Ito S, Matsushita T, Okamoto M, Suzuki T, Itoh M, Okumura-Noji K, Yokoyama S. Cholesteryl ester transfer protein and atherosclerosis in Japanese subjects: a study based on coronary angiography. *Atherosclerosis.* 2001;159:153–163.
64. Eiriksdottir G, Bolla MK, Thorsson B, Sigurdsson G, Humphries SE, Gudnason V. The -629C>A polymorphism in the CETP gene does not explain the association of TaqIB polymorphism with risk and age of myocardial infarction in Icelandic men. *Atherosclerosis.* 2001;159: 187–192.
65. Liu S, Schmitz C, Stampfer MJ, Sacks F, Hennekens CH, Lindpaintner K, Ridker PM. A prospective study of TaqIB polymorphism in the gene coding for cholesteryl ester transfer protein and risk of myocardial infarction in middle-aged men. *Atherosclerosis.* 2002;161:469–474.